Modulation of T lymphocyte activation by ORMDL3

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Als avis, en especial a l'àvia Montse
Als meus pares, Amado i Montse
a la meva germana, Carla
tot això és per a vosaltres.

“Los errores despiertan la capacidad para aprender y adormecen la autoestima”

JORGE WAGENSBERG

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Abstract

Genome wide association studies (GWAS) have pointed out *ORMDL3* gene as a risk factor for several proinflammatory and autoimmune diseases. The protein encoded by this gene belongs to a family of transmembrane proteins of the endoplasmic reticulum involved in calcium homeostasis and cellular lipid metabolism. The driving force behind this work was the compelling idea of finding out a precise mechanism for the pathological association of this protein. This thesis explores the potential role of ORMDL3 in T lymphocytes focusing on the activation process. Thus, we have demonstrated that calcium signaling and activation of T cells are influenced by the expression levels of ORMDL3. Besides, we have shown that inherited components of our genome modify ORMDL3 expression levels and lymphocyte physiology. Finally, we have characterized the molecular complex formed by ORMDL proteins. Altogether, this work allows a better understanding of the pathophysiology associated to ORMDL3 and its linkage with the immune system.

Resum

Estudis d'associació genètica ample han apuntat cap al gen *ORMDL3* com a factor de risc per diverses malalties pro-inflamatòries i autoimmunes. La proteïna codificada per aquest gen pertany a una família de proteïnes transmembrana del reticle endoplàsmic involucrada en l'homeòstasi de calci i en el metabolisme lipidic cel·lular. El motiu que ens va impulsar a dur a terme aquest treball era la idea de trobar el mecanisme darrere les associacions a patologia per aquesta proteïna. Aquesta tesis explora el rol potencial d'ORMDL3 en limfòcits T, amb èmfasi al procés d'activació. Així doncs hem demostrat que la senyalització de calci i l'activació de cèl·lules T es veu influenciada pels nivells d'expresió d'ORMDL3. A més hem demostrat que components del nostre genoma modifiquen els nivells d'expressió d'ORMDL3 i la fisiologia limfocitària. Per acabar hem caracteritzat el complex molecular de les proteïnes ORMDL. En conjunt, aquest treball permet una millor comprensió de la fisiopatologia associada a ORMDL3 i la seva relació amb el sistema immune.

Prologue

Genome Wide Association Studies (GWAS) approach is an excellent tool to find new genes implicated in diseases because it works without pre-candidate genes. However, sometimes the identified genes have unknown functions like the case of *ORMDL3*. At the beginning of this project there were few papers describing the function of this protein and even fewer associating it to pro-inflammatory diseases. The participation of ORMDL3 in calcium homeostasis made us presume that it might be altering the immune system, since these cells are highly dependent on calcium signaling.

T lymphocytes are essential components of the immune system. Dysfunction of these cells are the cause of several immune related diseases like asthma, inflammatory bowel disease, diabetes type 1, ankylosing spondylitis and rheumatoid arthritis, to mention some where *ORMDL3* has been genetically associated to. The precise understanding of how T cells get activated and mediate pathological processes will promote the development of new therapies for these complex diseases.

This thesis describes how *ORMDL3* expression modifies T cell activation, its regulation at physiological level and its functional consequences. In addition we provide evidences of the existence of a genetic component affecting T cell function that might explain the pathophysiology underlying the immune-related diseases where this gene has been associated to.

Abbreviations

ADAM-8	A Disintegrin and metalloproteinase domain-containing protein 8
APCs	Antigen presenting cells
ATF2	Activating transcription factor 2
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATF6f	Fragment of activating transcription factor 6
C/EBPß	CCAAT-enhancer-binding protein beta
C1P	Ceramide-1-phosphate
Ca^{2+}	Calcium
CaM	Calmodulin
Ca^{2+}/CN	Calcium-Calcineurin
CAD	CRAC activator domain
CC	Chemokine CC motif
CD	Crohn's disease
CRAC	Calcium release-activated calcium
CREB	cAMP response element binding
CXC	C-X-C motif
DAG	Diacilglycerol
DKO	Double knock out
EGTA	Ethylene glycol tetraacetic acid

eIF2α	Alpha subunit of eukaryotic initiation factor 2
ER	Endoplasmic reticulum
GRP78	Glucose-regulated protein of 78 kDa
GSDMB	Gasdermin B
GWAS	Genome wide association studies
IBD	Immflamatory bowel disease
IL-13	Interleukin 13
IL-2	Interleukin 2
IL-4	Interleukin 4
IMM	Inner mitochondrial membrane
IP_3	Inositol 1,4,5-trisphosphate
IRE1	Inositol-requiring protein 1
IS	Immune synapse
KCa3.1	Potassium intermediate/small conductance calcium-Activated channel, subfamily N, member 4
KO	Knock out
Kv1.3	Voltage-gated potassium channel, shaker-related subfamily, member 3
MCU	Mitochondrial Calcium uniporter
MEF	Myeloid eIf-1-like factor
MHC	Major histocompatibility complex
MMP-9	Matrix metallo protease 9
NCX	Sodium-calcium exchanger
NF-AT	Nuclear Factor of Activated T cells

OMM	Outer mitochondrial membrane
ORMDL	Orosomucoid-like
PBC	Primary biliary cirrhosis
PERK	Double-stranded RNA-dependent protein kinase-like ER kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLCγ	Phospholipase C-γ
PMCAs	Plasma membrane Ca^{2+}-ATPases
POST	Partner of STIM
pSS	Primary Sjorgen's Syndrome
S1P	Sphingosine-1-phosphate
S2	Drosphilae shneider 2
SAM	Sterile α motif
SMAC	Supra molecular activation cluster
SNPs	Single nucleotide polymorphisms
SOCE	Store operated calcium entry
SPT	Serine palmitoyltransferase
SPTLC	Serine palmitoyltransferase long chain base
STAT6	Signal Transducer and Activator of Transcription 6
STIM	Stromal interacting molecule
T1D	Type 1 diabetes
Tc	Cytolitic T cell
TCR	T-cell receptor

Th	T helper cell
TM	Transmembrane
TRPC	Transient receptor potential cation channel
TRPM4	Transient receptor potential melastatin 4
UPR	Unfolded protein response
VDAC	Voltage-dependent anion channel
WGAS	Whole genome association studies
WT	Wild type
XBP1	X-box binding protein 1

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i. INTRODUCTION

1. ORMDL family

Orosomucoid-like *(ORMDL)* gene family was first described in 2002 by Hjelmqvist *et al*. while characterizing genes from the retinitis pigmentosa locus (*RP26)* at 2q31-q33[1]. In that region localizes *ORMDL1* and, by using it as a template, two other members of the family, *ORMDL2* (12q13.2) and *ORMDL3* (17q21.1), were found in the human genome.

These genes codify for small proteins, around 150 amino acids, sharing more than a 80% of sequence identity between different members. Besides, the *ORMDL* family is highly conserved between species where vertebrates share a high homology (99% homology between human and mice) and cluster as a different lineage compared to plants and yeasts (Figure 1).

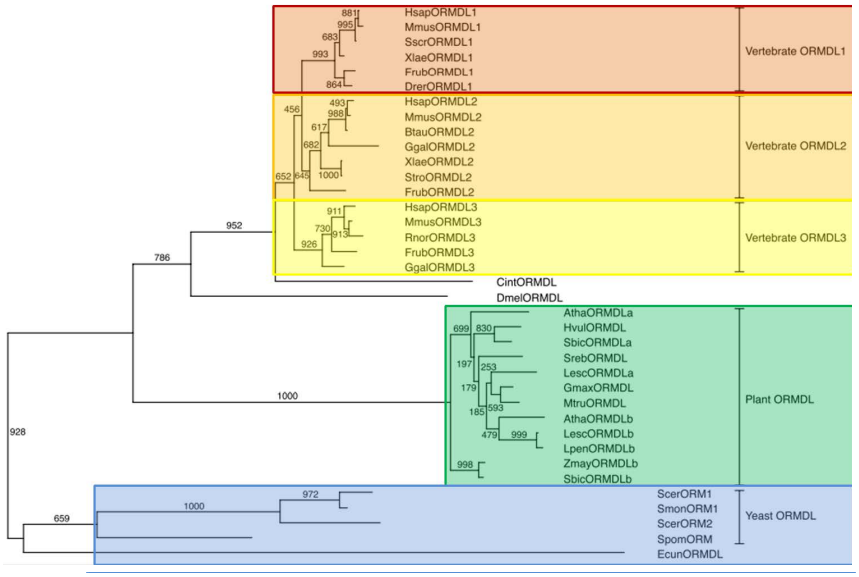

Figure 1: Phylogenetic tree of ORMDL protein sequence. Vertebrate ORMDL1 marked in red, vertebrate ORMDL2 in orange, vertebrate ORMDL3 in yellow. Plant ORMDLs are highlighted in green and yeast in blue. Abbreviations: Hsap (*Homo sapiens*, human); Mmus

(*Mus musculus*, mouse); Rnor (*Rattus novigerus*, rat); Sscr (*Sus scrofa*, pig); Btau (*Boss taurus*, cow); Ggal (*Gallus gallus*, chicken); Xlae (*Xenopus laevis,* African clawed frog); Stro (Silurana tropicalis*,* western clawed frog); Frub (*Takifugu rubripes,* Torafugu*)*; Drer (*Danio rerio,* zebrafish); Cint (*Ciona intestinalis,* sea squirt); Dmel, (*DrosophilaMelanogaster,* fruit fly)*.* Plants. Atha (*Arabidopsis thaliana)*; Hvul (*Hordeum vulgare)*; Sbic (*Sorghum vulgare)*; Sreb (*Stevia rebaudiana)*; Lesc (*Lycopersicon esculentum)*; Gmax (*Glycine max)*; Mtru (*Medicago truncatula)*; Lpen (*Lycopersicon pennellii)*; Zmay (*Zea mays)*; Yeast. Scer (*Saccharomyces cerevisiae*); Smon (*Saccharomyces monacensis*); Spom (*Schizosaccharomyces pombe*); Ecun (*Encephalitozoon cuniculi)*. Adapted from[1].

In this context it is important to highlight that there are only two members of the family in yeast, sharing around 35% of sequence identity with humans. Interestingly, the major differences come from the absence in mammalian ORMDLs of the amino terminal tail present in yeasts[1].

Human *ORMDL1*, *ORMDL2* and *ORMDL3* are ubiquitously expressed in fetal and adult tissues with decreased transcript levels on adult skeletal muscle, heart, lung and brain[1,2]. There are other works focusing on the third member of the family, *ORMDL3*, demonstrating an increased expression on lymphocytes and peripheral blood samples when compared to colonic epithelium, lung, brain and skeletal muscle[3,4]. In addition, this third member of the family has an alternative splicing isoform named ORMDL3 V1 that lacks exon number 2. This deletion implies a loss of 59 aminoacids on the amino terminal part and its tissue distribution resembles to the canonical ORMDL3[5].

1.1. Location and Structure

Immunostaining studies using heterologous expression systems and antibodies against the endogenous proteins in different cell types show that ORMDLs are resident in the endoplasmic reticulum[1,3,6–9] . Regarding their structure, there is controversy on the number of transmembrane segments crossing the ER membrane[1,2]. Howbeit, there is a common agreement on the cytosolic exposure of both amino and carboxy-terminals. Our laboratory proposes a two transmembrane model with an ER luminal loop, based on fluorescence protease assays and hydrophobicity plot analysis (Figure 2)[7].

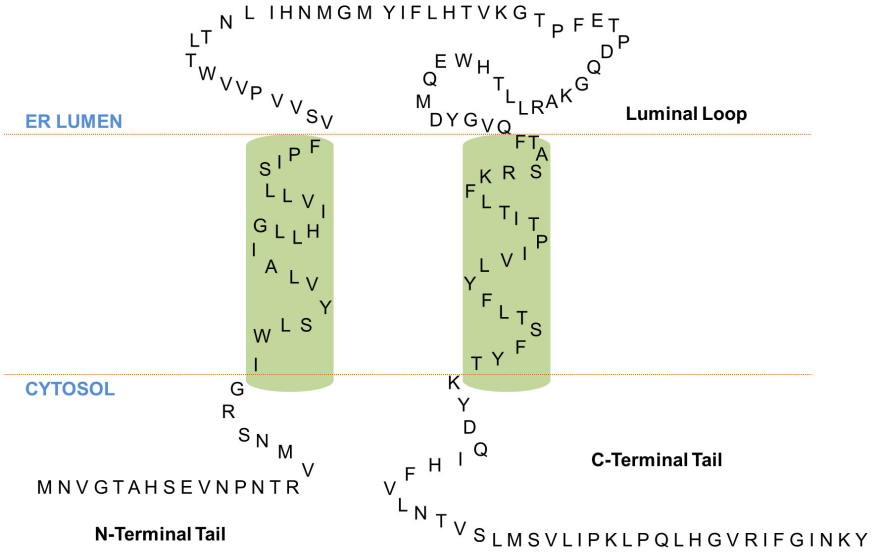

Figure 2: Representation of ORMDL3 structure in the ER with the aminoacidic sequence. N and C terminal tails face the cytosol, allowing an ER luminal loop.

1.2. ORMDL function

During many years the function of these proteins remained unknown. However, the third member of the family, ORMDL3, captured the attention of the scientific community when it showed up to be linked to several common diseases like asthma, inflammatory bowel disease, diabetes type I or rheumatoid arthritis by genetic association studies[3,10–21]. Since then, several papers have described changes in cell physiology associated to changes in ORMDL expression levels. This family has been implicated in three major processes: *de novo* sphingolipid synthesis, calcium (Ca^{2+}) handling and unfolded protein response (UPR).

1.2.1. ORMDLs modulate *de novo* sphingolipid synthesis

1.2.1.1. *De novo* sphingolipid synthesis

Intracellular sphingolipids come from different sources; extracellular uptake, turnover from complex sphingolipids and *de novo* synthesis (Figure 3). *De novo* sphingolipid synthesis is widespread among tissues and cell types. The first enzyme and rate limiting step to generate complex sphingolipids *de novo*, serine palmitoyltransferase (SPT), condensates a serine and a palmitoyl-CoA to form 3-Keto-sphinganine, becoming an interesting target to control the pathway[22]. This enzyme has two main subunits, serine palmitoyltransferase long chain 1 and 2 (SPTLC1 and SPTLC2).

The most studied sphingolipids derived from SPT function are ceramide (Cer), sphingosine (SP), sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P) and lyso-sphingomyelin (L-SM),

which have roles in the regulation of cell growth, death, senescence, adhesion, migration, inflammation, angiogenesis and intracellular trafficking (reviewed in[23,24]). In this respect there are several stimuli that modify the activity of this enzyme, summarized in Table 1. However, the pathway followed to regulate SPT is not well understood. This enzyme is not only regulated at transcriptional level but also by posttranslational modification and interaction with endogenous inhibitors such as the ORMDL proteins.

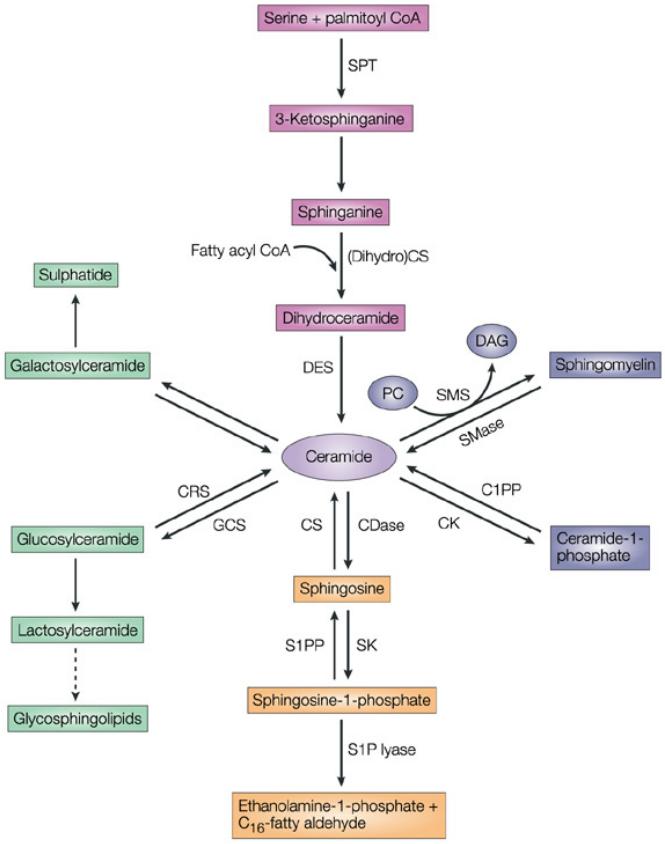

Figure 3: Synthesis of ceramide. This diagram shows different ways to produce ceramide. In pink it is highlighted the *de novo* sphingolipid synthesis. Hydrolysis from cerebrosides is marked in green, from sphingomyelin in blue and from ceramidases in orange. Obtained from[25].

Table 1

Stimulus	Tested	SPT fold activity	Ref
UVB	mouse epidermis and cultured human Keratinocytes	1.5	26,27
UVA	human keratinocytes	2.5	28
Endotoxin, IL1	liver, spleen and kidney of Syrian hamster	2 to 3	29,30
Endotoxin, IL1, tumour necrosis factor-α	human HepG cell line	2 to 3	29
Leptin receptor mutation	rat pancreas islet	Increase	31
Fatty acids	rat pancreas islet	Increase	31
Palmitic acid	rat astrocytes	1.3	32
Nicotineamide	cultured human keratinocytes	1.2	33
Etoposide	human leukemia Molt-4 cell line	2 to 3	34
Retinoic acid	mouse teratocarcinoma PCC7-Mz1 cell line	3	35
Resveratrol	human breast cancer cells	1.5	36
D^9-Tetrahydrocannabinol	a subline of the rat glioma C6 line	6	37,38
Activation of angiotensin II type 2 receptor	a subline of the rat pheochromocytoma PC12 cell line	2	39
N-4-hydroxyphenylretinamide	human neuroblastoma CHLA-90 cell line	2	40
Hexachlorobenzene	rat liver	1.5 to 2	41
Apolipoprotein E knockout	mouse liver	2	42

Table 1: Stimulus that modify SPT activity. This table has been adapted and modified from[43] and [44].

1.2.1.2. ORMDLs inhibit *de novo* sphingolipid synthesis

Most of the experiments exploring the role of ORMDLs in sphingolipid metabolism have been performed in yeast. There are two main evidences to demonstrate this function: i) double KO strain of endogenous Orms (ORMDL yeast ortologs) showed increased production of sphingolipids. ii) these proteins co-immunoprecipitated with yeast SPT subunits[45,46]. Since the first two works on 2010, the involvement of the yeast ortologs Orm1/Orm2 on SPT activity has been extensively characterized (summarized in Figure 4).

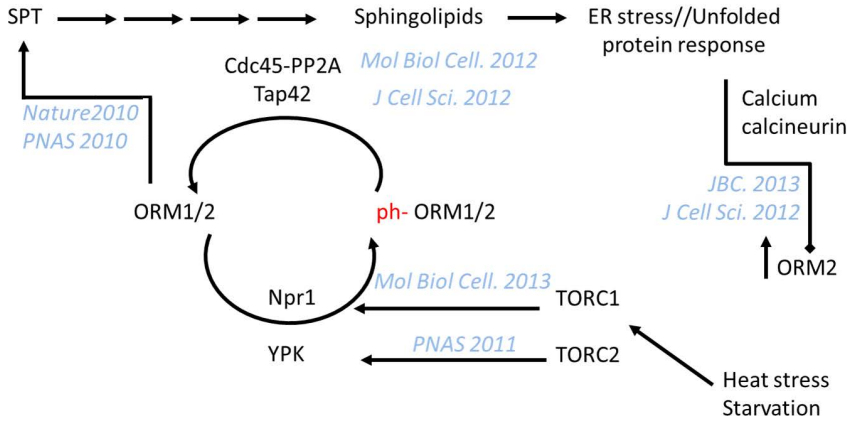

Figure 4: Orm1 and Orm2 participate in sphingolipid homeostasis. Orm1/2 inhibit SPT and reduce sphingolipid synthesis. The mechanism of regulation for these proteins implies a phosphorylation mechanism controlled by kinases and phosphatases downstream of TORC proteins. Another regulation mechanism is a Ca^{2+}/CN dependent up-regulation of Orm2.

Under low sphingolipids levels Orm1/Orm2 are phosphorylated and dissociated from SPT, thus leaving it on an active state to recover

lipid normosis[45]. There are 2 kinases responsible for Orm1/2 phosphorylation, YPK, that in turn is controlled by TORC2[47] and Npr1 downstream TORC1[48]. On the counterpart, there are two sets of phosphatases that can dephosphorylate Orm1/2. On one hand Tap42 phosphatase complex, downstream TORC1[49] and on the other Cdc45-PP2A under heat stress response[50]. Phosphorylation occurs in the N-terminus of ORMDLs and disrupts their oligomerization[45]. However, it is important to remark that this regulatory region is absent in mammalian isoforms.
Interestingly, this pathway is also regulated in yeast at the transcriptional level. It has been shown that these proteins are up-regulated under stress conditions[49] through a calcium-calcineurin (Ca^{2+}/CN) mechanism[51].

Regarding human ORMDLs' function and *de novo* sphingolipid synthesis, there are some evidences for a conserved role of these proteins among different species. The first evidence comes from the demonstration that human ORMDL3 can ameliorate growth defects on the yeast stain of *S. Cerevisiae* double KO for Orm1/2[1,45] and co-immunoprecipitates with SPTLC1 in a mammalian system[45]. The second followed a similar approach than the one performed in yeast. Thus, down-regulation of the three ORMDL members enhances SPTLC activity in HeLa cells. Suggesting therefore, a redundant function for the three members since the presence of any of them was enough to mediate *de novo* sphingolipid synthesis inhibition[52]. However, there are no evidences demonstrating the impact of individual overexpression of the ORMDL family members on SPTLC function.

1.2.2. ORMDLs, ER stress and unfolded protein response

1.2.2.1. ER stress and unfolded protein response

The ER is a cellular organelle with many functions related to protein folding, calcium store and lipid synthesis among others. ER stress is defined as any cellular state in which the folding capacity of the ER is overwhelmed owing to an increase in protein load and/or disruption of the folding capacity[53]. Under ER stress the cell activates signalling pathways to overcome this situation. The events that occur in response to ER stress are named Unfolded Protein Response (UPR).

UPR is composed of three principal branches. The stress sensors are: inositol-requiring protein 1 (IRE1), double-stranded RNA-dependent protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6)[54]. All of them get activated with a similar mechanism. Under ER stress, the ER-resident chaperone glucose-regulated protein of 78 kDa (GRP78), also known as BiP, activates the UPR sensors by dissociation form their ER luminal domain[55,56]. Figure 5, resumes ER stress activation and UPR pathways.

PERK gets activated and promotes global protein synthesis inhibition via phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α)[57]. Not all translation processes are reduced during this event, on the contrary some increase their levels, like the transcription factor ATF4, which activity is important for aminoacid metabolism and redox state[57–59].

Another ER stress sensor is ATF6. Under ER stress GRP78 dissociates from ATF6 and translocates to the Golgi. In the Golgi,

site-1 (SP1) and site-2 (SP2) proteases process ATF6 releasing its cytosolic fragment (ATF6f)[60,61]. ATF6f goes to the nucleus and acts as a transcription factor that controls genes that encode for ER-associated protein degradation (ERAD) machinery and ER chaperones[60–62].

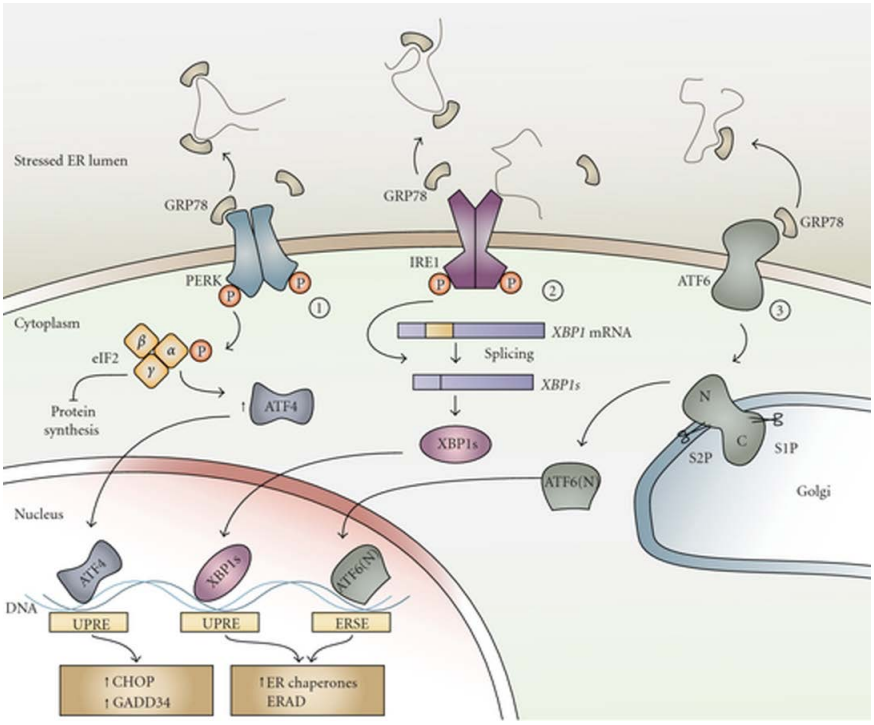

Figure 5: ER stress and UPR pathways. Alterations on ER homeostasis triggers UPR to overcome the stress situation. Adapted from[63].

ER stress promotes IRE1 trans-autophosphorylation, which activates the RNAse activity of its cytosolic tail. This RNAse domain triggers the unconventional splicing of *Xbp1* mRNA (X-box binding protein 1) resulting on *Xbp1s*, that once translated to XBP1s acts as a transcriptional factor that regulates many UPR target genes[60,64–66].

1.2.2.2. ORMDLs and UPR

ORMDLs are related to ER stress and UPR events. The first report bridging ORMDLs and stress signals was performed by Hjelkvist *et al.* in 2002 where DKO yeast strains of *S.Cervisiae* for Orm1 and Orm2 treated with ER stressors, Tunciamycin and Dithiothreitol, had a growth impairment[1]. This fact has also been shown in yeast by other groups[45,46,49] indicating an impaired folding function in this organelle.

Interestingly, in 2008, Araki *et al*. showed in mammalian cells that ORMDLs triple knock down presented an impaired maturation of nicastrin, a component of the gamma secretase complex, suggesting that the absence of ORMDLs challenge the proper protein folding and/or stability[2].

There are several works where the impact of ORMDL3 expression on UPR pathways has been addressed attempting to explain the pathophysiology associated to this protein. However, contradictory results were obtained depending on the cell model and technical approaches.

Our lab has shown that ORMDL3 overexpression is linked to an increase on the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α), suggesting a role on PERK activation[7]. Another branch of the UPR, ATF6, is also claimed to be related to ORMDL3. Two studies from the same group claim that overexpression of ORMDL3 on lung epithelial cells (A549 cells) induces ATF6 nuclear translocation. Moreover a transgenic mice overexpressing ORMDL3 showed also an increased ATF6 nuclear localization on bone marrow derived macrophages[69]. On the contrary, in 2010 McGovern *et al.* described a decreased transcriptional activity of UPR elements after treatment with the ER

stressors tunicamycin and thapsigargin in cells overexpressing ORMDL3[3]. Finally, another study that tested the 3 branches of UPR on 1HAE and A549 cells reported no changes in cells with different ORMDL3 levels[67].

1.2.3. Calcium homeostasis and ORMDL3

1.2.3.1. Calcium homeostasis

Calcium ion has a dual role in cell physiology. On one hand, calcium determines the membrane potential and cell excitability acting as a divalent cation. On the other hand, it acts as an important secondary messenger. These unique properties allow calcium to participate in many biological functions like fertilization, development, metabolism, secretion, muscle movement, neuronal activity, programmed cell death or necrosis.

The way calcium signalling works is based on the establishment of a calcium gradient between the extracellular and intracellular compartment that allows the generation of cytosolic calcium peaks with different outcomes depending on time, concentration, and pattern. At resting states, cytosolic calcium levels are low (nM range) and under a specific stimuli membrane channels open increasing cytosolic [Ca^{2+}] in order to promote a cellular change. The mechanisms that increase calcium levels are named ON mechanisms whereas those decreasing it are named OFF mechanisms (Figure 6)[68].

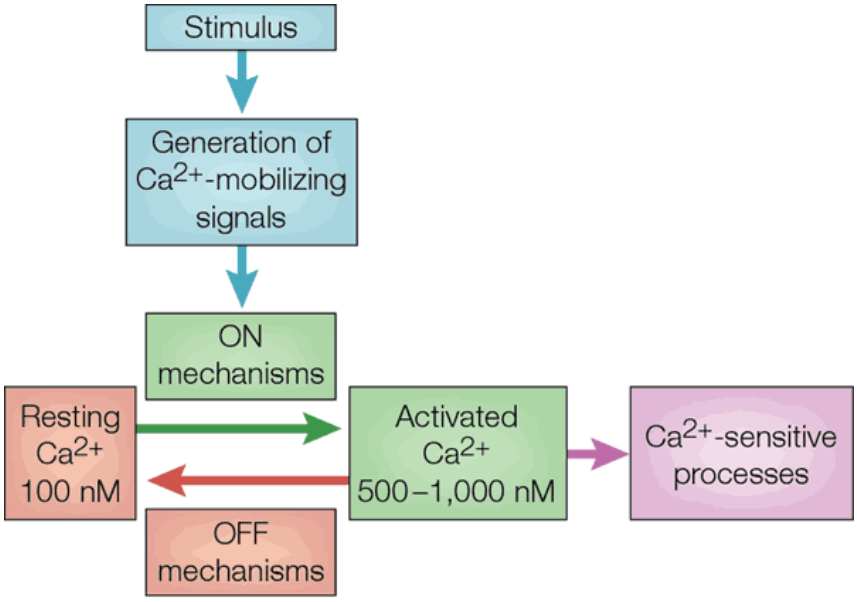

Figure 6: Calcium handling in the cells. ON mechanisms take advantage of the electrochemical gradient whereas off mechanisms require energy expenditure to be performed. Adapted from[68].

There is an energetic cost to maintain low cytosolic calcium levels and several pumps and transporters work against electrochemical gradients to maintain them[69]. Different mechanisms can modify cytosolic-free calcium levels: changes in calcium binding proteins buffering capability in the cytosol and ER lumen; changes in the activity of calcium channels, calcium transporters, calcium ATPases present in the plasma membrane or within intracellular stores like ER or mitochondria[70].

In summary, calcium signalling is a tight regulated event on the cell, due to its relevance in many processes and to the versatility that comprises.

1.2.3.2. ORMDL3 expression modulates calcium homeostasis

In 2010, our laboratory demonstrated that ORMDL3 is involved in calcium homeostasis[7]. The studies, performed in heterologous expression systems, showed that ORMDL3 expression correlated inversely with ER calcium content. This calcium imbalance is due to an inhibitory effect of ORMDL3 on SERCA pump activity promoting increased cytosolic calcium levels[7] (Figure 7). Interestingly it has been also described a transcriptional regulation of SERCA depending on ORMDL3 expression levels[6,9].

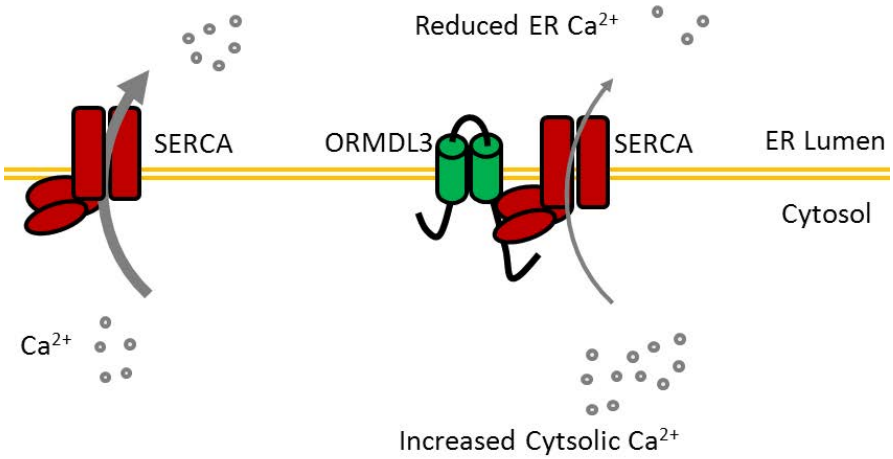

Figure 7: ORMDL3 binds to and inhibits SERCA pump. This produces an increase in cytosolic Ca^{2+} levels and a reduction on ER Ca^{2+} content.

Finally, there is a work that reinforces the idea that ORMDL3 acts as a break of SERCA to buffer and clear cytosolic calcium levels and at the same time explores the consequences in immune cells, specifically eosinophils. Thus, the knock down of ORMDL3

expression showed decreased cytosolic calcium levels and impairment in migration and degranulation of these cells[8].

1.3. Genome wide association studies and ORMDL3

Genome wide association studies (GWAS), also known as whole genome association studies (WGAS) are based on a comparison between two populations, with hundreds to thousands of individuals per group, one that has a trait, or a disease, and a control one. The genome of these two populations is sequenced and single nucleotide polymorphisms (SNPs) are evaluated. If one allele in a SNP is more frequent in one population than the other, it is assumed that that allele is associated with the trait or disease tested in the study[71]. GWAS have achieved in the recent years two main goals: confirming existing studies performed by candidate gene approaches with bigger cohorts; and providing new candidate genes of known or unknown function as risk factors for several diseases. Using this technique, in 2007 *ORMDL3* was the first gene to be associated to childhood asthma[4]. Concretely, a correlation between SNP rs7216389 and childhood asthma presented increased transcript levels for *ORMDL3* [4].

To date, other studies with different populations have replicated what Moffatt *et al.* showed in 2007[72–78]. Moreover, other GWAS and genetic studies have related *ORMDL3* and the chromosomal zone where it is 17q12-21, not only with asthma, but to other diseases and parameters like Crohn's disease[11,12,15], ulcerative colitis[3], primary biliary cirrhosis[79–81], diabetes type 1[10], white blood cell count[82], rheumatoid arthritis[13], alergic rhinitis[83], glioma[84], and cervical cancer[85] (for a detalied description of the region see Apendix I). Many of these linkage studies may lead to the thought

that this zone is important for immune system's related complex diseases.

The most extensively studied SNP is rs7216389, the one that was first described and linked to asthma by Moffatt *et al.* on 2007. Despite it is placed in an intronic zone of *gasdermin B* gene (*GSMDB*), the change, (C/T) modifies *ORMDL3* transcription and might affect the binding site of the proimmflamatory transcription factor CCAAT-enhancer-binding protein beta (C/EBPß). This fact would also suggest a link between ORMDL3 expression levels associated to this SNP and the immune system physiology[4].

1.3.1. Asthma disease and ORMDL3

Asthma is an inflammatory disorder of the airways that affects between a 1 and 18% of the population; characterized by a reversible obstruction of the airflow[86]. This disease is defined as a common chronic disorder of the airways that involves a complex interaction of airflow obstruction, bronchial hyper responsiveness, and an underlying inflammation[87].

It is known that the immune system's profile in asthma is characterized by an imbalance on the response of Th1 and Th2 lymphocytes that modulates airways muscular contraction as well as pulmonary remodelling due to chronic inflammation[88,89]. Nevertheless there are other cell types involved in asthma pathophysiology like: eosinophils [90], epithelium [91], neuronal, and muscular cells [92,93].

The causes of asthma are environmental and genetic. Regarding the genetics of asthma revealed by GWAS there are several loci in or near the genes *CHI3L1* (also known as *YKL40*), *IL6R*, *DENND1B*, *IL1RL1–IL18R1*, *PDE4D*, *RAD50–IL13*, *HLA-DQ*, *IL33*, *SMAD3*, *ORMDL3–GSDMB*, *IL2RB,* and *PYHIN1*. Surprisingly, most of these loci reported in one GWAS have not been replicated in other studies with the exception of the SNPs related to *ORMDL3–GSDMB*, which are related to childhood asthma and to adult asthma (Appendix I) [18,94–98].

Taking in consideration the reproducibility of the association between *ORMDL3* gene and asthma, most of the works exploring ORMDL3's pathophysiology have focused on this disease as a model of study. Here we summarize the most important findings:

i) Induction of allergic asthma in a mouse model promotes increased ORMDL3 expression in the epithelium. In the same work the authors demonstrated that by modifying ORMDL3 levels in an airway cell line, there was an alteration of the cellular transcriptional program towards a more pro-inflammatory one; affecting for example the expression of metallo-proteases (MMP-9, ADAM-8), chemokines (CCL-20), chemokines (IL-8, CXCL-10, CXCL-11), OAS genes and calcium pumps (SERCA)[6]. In contraposition to this study, in 2013 Hsu KJ *et al.* manipulated the levels of ORMDL3 on normal human airway epithelial cells to further evaluate UPR parameters and innate immunity responses and found no differences. This work argued against a role of ORMDL3 modulating innate immune responses carried out in the lung epithelium[67].

ii) The role of ORMDL3 in eosinophils has been also explored in the context of allergic inflammation. Murine eosinophils that migrate to the airways after allergic challenge presented high levels of ORMDL3 expression. Interestingly, in this work the authors demonstrated that ORMDL3 was relevant for trafficking, recruitment and degranulation of eosinophils[8].

iii) Another link between asthma and ORMDL3 is the relationship of ORMDL3 and the inhibition of *de novo* sphingolipid synthesis. To understand the relationship of asthma and sphingolipid synthesis, Worgall *et al.* used two approaches. On one hand, myriocin (Myr), an inhibitor of SPT, was administered by inhalation to wild type (WT) mice. The other approach used SPTLC2 heterozygous knockout (KO) mice. Interestingly, both approaches produced a decreased *de novo* sphingolipid synthesis, which in turn, increased bronchial reactivity in the absence of inflammation. The authors justified this increased hyper-reactivity due to an imbalance for magnesium homeostasis on the bronchia[99].

iv) Finally, a transgenic mice constitutively overexpressing ORMDL3 protein on all tissues has been studied in the context of lung pathology. This mouse develops asthma spontaneously. This phenotype might be mediated by UPR activation, concretely the ATF6 axis. Besides, the authors argued that airway remodelling preceded immune cell infiltration in this transgenic model, supporting the idea that ORMDL3 pathophysiology in the airways comes from the epithelium[9].

1.4. Regulation of mammalian ORMDL3 expression

ORMDL3 promoter is fully un-methylated independently of allelic differences. However, regions 31Kb away from *ORMDL3* may exert enhancer activity because they are susceptible to allele specific regulatory effects and epigenetics like nucleosome occupancy and DNA methylation[100,101].

Figure 8: Description of the minimum promoter region for human ORMDL3. Schematic representation of *ORMDL3* gene with its 3 exons within the transcript. When amplifying the promoter it can be seen 3 binding sites for Ets-1, P300 and CREB described in [102]. Bottom, minimum promoter sequence (-64/-56) contains a STAT6 binding motif that acts in conjunction with P300[103].

There are 2 works addressing human ORMDL3 promoter function. On one hand a work in 2012 by Jin R *et al*. claims that ORMDL3 promoter is at position -84/+58 taking as reference the transcription start sequence (TSS). Mutation analysis of this region revealed that Ets-1, p300 and CREB were controlling ORMDL3 expression[102]. In agreement, Qui R *et al.* (2013), using a similar approach for ORMDL3 promoter's characterization with luciferase assays, discovered the minimum promoter sequence on -64/-56 position from the TSS. This region contains a STAT6 binding motif that is

claimed to act in conjunction with P300 (Figure 8). STAT6 activity is induced under interleukin 13 (IL-13) or interleukin 4 (IL-4) pathways in agreement with the ORMDL3 induction seen under this treatments. The role of STAT6 pathway for ORMDL3 induction was further confirmed with a STAT6 KO mouse[6].

Another pathway that seems involved in ORMDL3 expression regulation in mice is cAMP/PKA/CREB[104,105].

2. Ca^{2+} and T cell activation

T lymphocytes are white blood cells that play a central role in cell-mediated immune responses. There are different subsets of T cells based on their function, membrane expression markers, and protein secretion. T cells can be divided in two main groups, helper (Th) and cytolitic (Tc) depending on the surface expression of CD4 or CD8 glycoproteins, respectively. T cells get activated upon the binding of the T cell receptor (TCR) to the antigen bound to the major histocompatibility complex I or II (MHC class I or II) present on antigen presenting cells (APCs). Upon activation T cells undergo proliferation and differentiate in order to participate in the immune response[106].

The relevance of calcium on immune cells can be divided in two temporal behaviours. On one hand an acute and short-termed Ca^{2+} entry is necessary for mast cell degranulation and for effective cytolitic activity of T cells (reviewed in[107,108]). On the other, long term effects of Ca^{2+} rely on the nuclear translocation of nuclear factor of activated T cells (NF-AT) and the transcription of its target genes for a proper T cell differentiation from naïve to the different T cell subsets.

The major Ca^{2+} entry pathway in T lymphocytes is a high selective Ca^{2+} channel placed in the plasma membrane. This channel, known as calcium release-activated calcium (CRAC) channel mediates store operated calcium entry (SOCE).

This section will focus on the molecular mechanisms that underlie the CRAC current and its involvement in T cell activation.

2.1. Calcium handling during T cell activation.

Peripheral T cells are circulating around the organism in a state named naïve T cell. This lethargic state is prolonged until they find an antigen that activates them[109,110]. The interaction of APCs and T cells induces the rearrangement of different proteins of the plasma membrane to form what is known as the immune synapse (IS)[111].

The IS presents a cytoskeletal and membrane rearrangement towards the contact between the T cell and the APC, creating a supra molecular activation cluster (SMAC) with high amounts of adhesion molecules and antigen receptors[112]. Interestingly CRAC channels are placed in the IS[113,114].

Upon TCR antigen stimulation, a rise in cytosolic Ca^{2+} takes place due to the release of Ca^{2+} from the ER and the opening of CRAC channels. CRAC channels are gated as a consequence of ER calcium depletion that occurs during the activation cascade, a process known as SOCE. Calcium binding to calmodulin (CaM) activates the phosphatase calcineurin (CN). At resting conditions, NF-AT is hyper-phosphorylated and retained in the cytosol but under CN activation, NF-AT gets dephosphorylated and shuttled into the nucleus to exert its activity as transcription factor[115].

The machinery involved in calcium entry during T cell activation implies a coordinated work of different organelles around the IS in order to keep and shape a sustained calcium entry and avoid calcium-dependent inactivation of CRAC channels, a negative feedback present in this pathway. In the plasma membrane there are Ca^{2+}-ATPases (PMCAs) that extrude Ca^{2+} towards the extracellular space. Placed in the ER, SERCA pumps clears calcium in the vicinity of CRAC channels towards the lumen of this organelle[116]. A similar mechanism occurs in mitochondria near the

IS. These organelles, are the second bigger Ca^{2+} store in T cells and buffer the cytosolic calcium increase by the coordinated work of the voltage-dependent anion channel (VDAC), in the outer mitochondrial membrane (OMM)[117], and the mitochondrial Ca^{2+} uniporter (MCU) placed in the inner mitochondria membrane (IMM)[118]. Finally, another important mechanism for cytosolic Ca^{2+} removal is the sodium-calcium exchanger (NCX) that uses the electrochemical gradient of Na^{+} to extrude calcium[119].

2.2. Store operated calcium entry

Upon TCR stimulation and IS formation, adhesion molecules and serine-threonine kinases target the plasma membrane. As a result phospholipase C-γ (PLCγ) gets phosphorylated and activated[121]. PLCγ activity at the plasma membrane cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacilglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 diffuses towards the cytosol and gates IP_3 receptors, Ca^{2+} channels placed in the ER that upon opening allows Ca^{2+} efflux towards the cytosol[122]. Finally, store depletion promotes an extracellular Ca^{2+} entry via CRAC channels[123] (Figure 9).

The molecular identity of the CRAC channel has been elusive for a long time. However, in the last years many advances have been done revealing its molecular identity and function in the immune system. Using siRNA screening in drosophila schneider *2* (S2), and human cells, and testing for calcium entry after store depletion with the SERCA inhibitor thapsigargin two families of proteins that impaired SOCE were identified. On one hand stromal interacting molecule 1 and 2 (STIM1 and STIM2), on the other calcium

release-activated calcium channel 1 (CRACM1) also named Orai that has 3 independent genes[124–128]. The discovery of the CRAC channel identity has driven a huge amount of scientific works increasing our understanding of SOCE events.

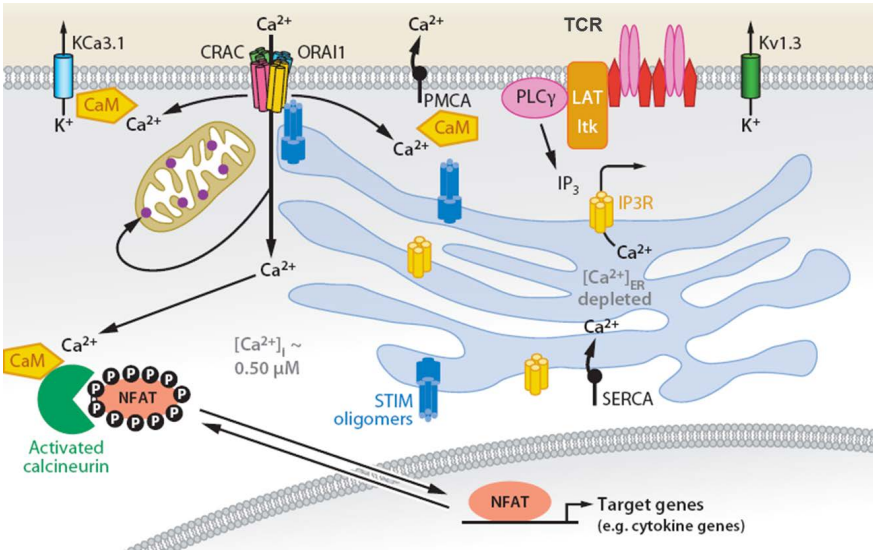

Figure 9: Ca^{2+} entry in activated T cells. Upon TCR activation PLCγ produces IP_3R activation in the ER. Ca^{2+} leaves the ER and stromal interacting molecules (STIM) oligomerize to activate Orai1. This Ca^{2+} promotes CaM/CN/NF-AT activity and gene transcription. Extrusion mechanisms are mediated by PMCA, SERCA and also mitochondria (MCU, violet spots). Potassium channels maintain the hyperpolarization of the membrane to maintain Ca^{2+} influx. Obtained from[120].

2.2.1. The CRAC channel

CRAC currents had been discovered using electrophysiological recording techniques long before the discovery of STIM and Orai proteins. The most studied models that have given detailed information regarding the characteristics of CRAC current are T

cells, mast cells and hematopoietic-like cell lines. The current developed after store depletion in these systems is named $I_{CRAC.}$

2.2.1.1. Channel gating: STIM

STIM proteins are encoded by two genes, *stim1* and *stim2* that upon translation present mainly an ER resident pattern[129]. STIM1 is the most widely studied activator of CRAC channel and exerts two principal functions: first, to sense the store Ca^{2+} content and second, to communicate this information to the channels present in the plasma membrane.

Nuclear magnetic resonance (NMR) studies of the ER luminal fragment of STIM1 reveal two EF-hands followed by a sterile α motif (SAM)[130,131]. This domain has a low affinity for Ca^{2+} binding (K_d 500-600μM)[132] in agreement with the range of estimated ER luminal concentration. Upon store depletion and Ca^{2+} dissociation the ER luminal part promotes the oligomerization of STIM1. These oligomers redistribute in the ER membrane to form puncta, a reversible dotted pattern nearby the plasma membrane in a Ca^{2+}-dependent manner[133,134]. It is believed that binding to Orai1 maintains STIM1 in this close distance to plasma membrane, moreover plasma membrane phospholipids that also bind STIM1 are thought to be important to maintain puncta[135].

The way STIM1 gates Orai1 is mediated via a domain in the cytosolic tail of STIM1 named CRAC activator domain (CAD). This domain binds directly to the carboxy-terminal tail of Orai1 and opens it (Figure 10)[136,137].

Other mechanisms for Orai1 gating that do not imply store depletion have been described. Orai1 activation by STIM1 has

been seen under hypoxia, acidification, oxidative stress and temperature change[138].

Interestingly STIM1 is claimed to gate directly transient receptor potential cation channel family (TRPC) proteins. This group of proteins were candidates to be components of SOCE before the discovery of STIM and Orai proteins. A direct interaction has been claimed to gate TRPCs but it is still a matter of debate[139–142].

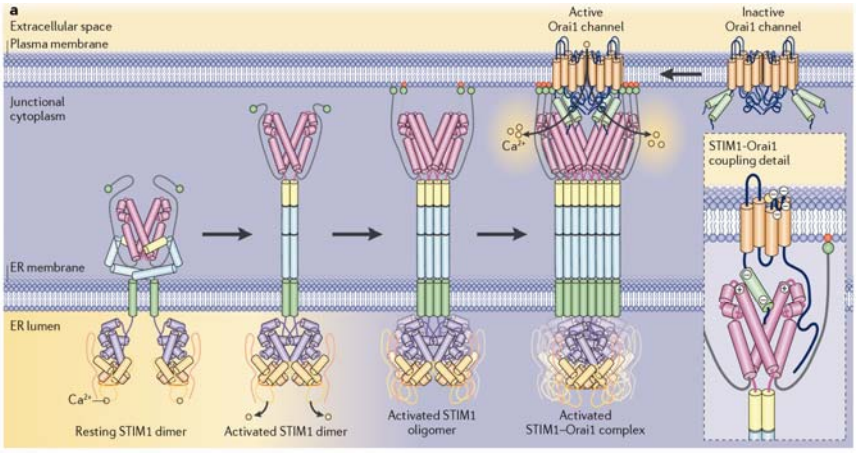

Figure 10: STIM1-Orai1 interaction. STIM1 oligomerizes upon store depletion, binds Orai1 and promotes channel gating. Obtained from[143].

2.2.1.2. ORAI: the CRAC pore

Orai family comprises 3 genes that encode for Orai1, Orai2 and Orai3. The three of them can be gated by STIM proteins, are located in the plasma membrane, and are widely expressed in different tissues[144].

Single Orai1 protein has four transmembrane domains with the amino and carboxy-terminal tails as well as the loop between transmembrane (TM) 2 and 3 facing the cytosol[120].

Functional Orai channel was thought to be formed by four monomers, forming a tetramer. Evidences supporting this conformation came from the observation that a dominant negative form of Orai1 (E106Q) was able to block CRAC currents in cells transfected with monomers, concatenated dimers and concatenated trimmers, but not concatenated tetramers[145]. However the crystal structure of the *Drosophila melanogaster* Orai1 recently revealed an hexameric conformation[146]. Interestingly, this conformation has less Ca^{2+} selectivity than the tetrameric one[147]. Sequence screening of ORAI1 and loss of function mutation-studies have revealed different residues relevant for channel function and Ca^{2+} selectivity (Figure 11).

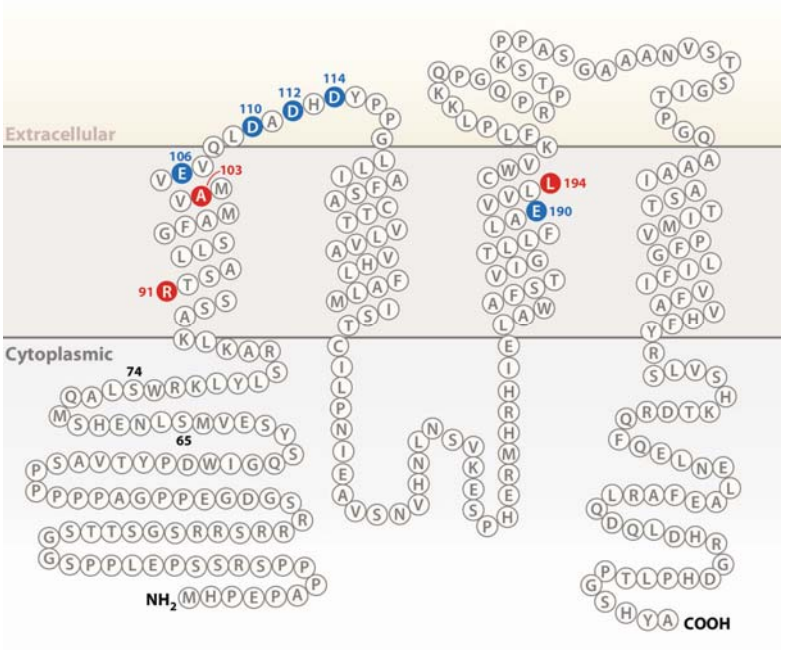

Figure 11: Aminoacid sequence of human Orai1. Residues that have been associated with human immunodeficiency are R91, A103 and L194, mutated to W, E and P, respectively. Other residues affect channel function and selectivity (E106, D110, D112 and E190). Adapted from[120].

2.3. The CRAC channel current and I_{CRAC}

Whole cell I_{CRAC} currents are typically small and their current-voltage relationships (I-V curves) show an inwardly-rectifying current-voltage relation with a reversion potential around +50mili volts (mV). CRAC current also displays a high selectivity for Ca^{2+} over monovalent cations and a lack of voltage dependence[148].
The high Ca^{2+} selectivity is deduced due to a dose dependent reversible inhibition of CRAC currents under replacement of Ca^{2+} on the extracellular solution by divalent cations. In addition, under removal of divalent cations, CRAC presents a conductance for Na^{+}, 1000 times lower than for calcium[149]. Another characteristic of this channel is the small unitary conductance that presents, thus, to date no single-channel CRAC currents have been reported. Noise analysis in whole cell configuration in Jurkat T cells has revealed a conductance for I_{CRAC} of 9-24 fS in 2-110mM external calcium[150,151]. Removal of divalent cations from the bath solution and recording of Na^{+} currents together with noise analysis revealed a conductance also very small, about 700 fS[152].

2.3.1. The CRAC channel fingerprint

Different aspects make I_{CRAC} unique. Chemical inhibitors and current behavior under different compounds unveiled the characteristics of this current even before STIM and Orai were discovered.

2.3.1.1. Chemical modulation

Despite there is no specific chemical described for I_{CRAC} modulation, some studies have explored different compounds known to modulate this channel (reviewed in[153]).

Endogenous lipid metabolites (sphingosines and ceramides) are known to block CRAC currents[154]. Another popularly used compound is 2-aminoethyldiphenilborae (2-ABP) that presents a dual role on CRAC currents. On one hand, low concentrations of 2-ABP (<5μM) enhances current density whereas increased concentrations has an inhibitory effect[155].

The list of inhibitors for CRAC is growing due to the great interest on controlling T cell activation. Compounds like: divalent and trivalent ions (La^{3+}, Gd^{3+}), capsaicin, 5-Nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), bistrifluoromethyl-pyrazole derivative (BTP2), diethylstilbestrol (DES), bromenol lactone (BEL), bile acids and 1-(5-Chloronaphthalene-1-sulfonyl) homopiperazine (ML-9) are reviewed in[156].

2.3.1.2. Ca^{2+} dependence of CRAC channels

Modulation by Ca^{2+} is also one characteristic of this current and two main phenomena are well described: Ca^{2+} dependent potentiation (CDP) and Ca^{2+} dependent inactivation (CDI) that is divided in two components; fast CDI (fCDI) or slow CDI (sCDI).

2.3.1.2.1. Calcium dependent potentiation

CDP implies a process where Ca^{2+} potentiates Orai opening. This idea comes, first, from the observation that the removal of

extracellular Ca^{2+} decreases CRAC activity and second, that upon store depletion and re-addition of extracellular Ca^{2+} to the external bath solution, Orai channels undergo conformational changes into an open state[153,157].

2.3.1.2.2. Fast calcium dependent inactivation

Fast CDI (fCDI) implies internal STIM1-Orai1 molecular interactions that induce an inhibition of the channel in the range of milliseconds upon activation. The common idea is that Ca^{2+} enters the channel and binds nearby the pore, thus producing its inactivation. fCDI can be monitored under hyperpolarization of the plasma membrane and with high Ca^{2+} in the extracellular solution. The relevance for Ca^{2+} in this process comes from the observation that development of the current under monovalent cations in the absence of external divalent cations presents no fCDI[158]. In agreement, the use of the fast chelator 1,2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA) in the internal solution reduces this fCDI when compared with a slower chelator like ethylene glycol tetraacetic acid (EGTA)[149,154]. Recent studies suggest that STIM1 has an inhibitory domain region (ID region) that if replaced prevents fCDI[159,160]. In addition, many works suggest that stoichiometry of STIM1-Orai1 interaction affects fCDI (reviewed in[161]).

2.3.1.2.3. Slow calcium dependent inactivation

The level of cytosolic Ca^{2+} in the cytosol is important for sCDI. This is deduced form the observation that using high concentrations of EGTA inside the pipette (12mM) prevents slow CDI whereas low concentrations of EGTA (1.2mM) do not. This places the

mechanism for slow CDI far from CRAC's pore. The same phenomena is observed when using BAPTA, a strong Ca^{2+} chelator that, when used inside the pipette prevents slow CDI.

Slow CDI has been related to the ability of different events nearby CRAC channel that can affect the averaged cytosolic Ca^{2+} levels. It is assumed that one of the functions of CRAC channels is to refill the depleted ER stores and that this is accomplished by SERCA ATPases present in puncta formations[162]. In support for this idea, Tg-mediated I_{CRAC} development implies a 50% of decrease in sCDI compared to IP_3 dialysis[163]. However induction of I_{CRAC} with the SERCA inhibitor Tg, also presents a slow inactivation of CRAC currents suggesting a store independent mechanism for sCDI[163,164]. The mechanism for this store independent sCDI has been attributed to mitochondrial activity in the surroundings of CRAC channel[165–167] (Figure 12).

2.3.2. Modulation of CRAC

After the identification of STIM1-Orai1 as the main CRAC channel unit, different studies have focused on its modulation mechanisms. Different proteins bind CRAC channel and affect its activity. However, direct interaction is not necessary for CRAC current modification, as other channels affect the electrochemical driving force for Ca^{2+} entry, as well as pumps and organelles that can affect the slow CDI (described above).

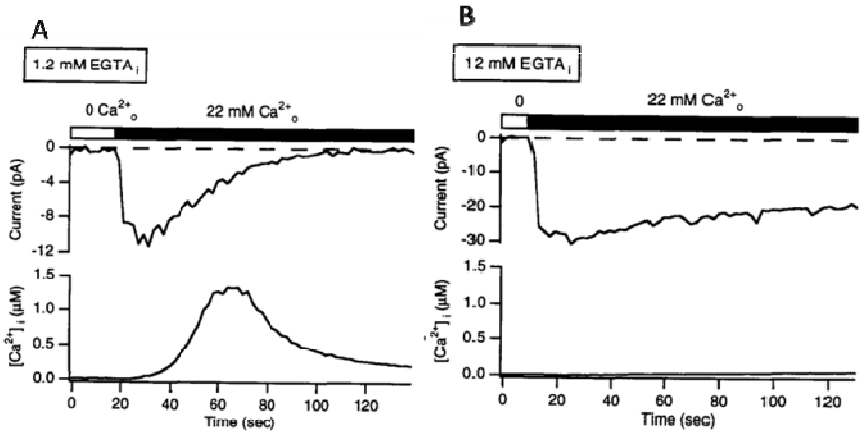

Figure 12: Slow inactivation studies on I_{CRAC}. A: I_{CRAC} presents a strong CDI after peak reach (second 20) and an inactivation on Ca^{2+} peak after second 60. B: Strong chelating conditions (12mM EGTA) prevents sCDI as there is no current decrease after peak. No Ca^{2+} is detected due to EGTA chelator[163].

2.3.2.1. Interacting partners

Different proteins have been related to bind either STIM1 or Orai1 affecting CRAC activity.

One of the first proteins described to affect CRAC channel activity by modulating fCDI was CaM[168]. CaM is thought to exert this function when binding to the N-terminal cytosolic tail of Orai1. This same region in Orai1 seems to be the binding site for CRAC2A, another cytosolic protein that stabilizes the interaction of STIM1-Orai1. This protein can decrease SOCE up to a 50% when interfered[169].

However, not only the binding of regulators to Orai1 is important for channel function. STIM1 interacting proteins have also the ability to

modulate CRAC. Golli was known to inhibit SOCE through its binding to STIM1 on its C-terminal tail[170,171]. In addition, SOCE-associated regulatory factor (SARAF) is an ER resident protein also associated to STIM1 and to inhibit SOCE via a slow calcium dependent inactivation (sCDI)[172]. Finally, the ER resident protein Calnexin (ERp57) binds to the luminal tail of STIM1 and modulates its sensitivity to store depletion[173].

2.3.2.2. Modulators of CRAC without a direct interaction

Different events during SOCE can modulate the amplitude and extent of CRAC current, here there is a resume of those that are relevant for T cell activation.

As mentioned before in the chapter of sCDI, SERCA pumps are important to refill the depleted stores and its inhibition implies a 50% of decrease in sCDI[164] due to a decreased ER refilling. In agreement to this idea SERCA is also present in puncta during store depletion being called the third member of puncta[162,174].

Another Ca^{2+} pump, PMCA, is claimed to play a role during CRAC channel activation. This plasma membrane pump is inhibited in the IS by STIM1, promoting a Ca^{2+} entry in the IS[175,176].

One protein that binds STIM1 but does not affect directly SOCE is a protein named partner of STIM (POST). This scaffold protein binds STIM1-Orai1 under store depletion and scaffolds SERCA pumps, PMCAs and NCX within STIM1-Ora1 juntions[177].

There are other ion channels that modify CRAC channel activity by affecting the driving force for calcium entry through CRAC channels. Thus, upon SOCE the amount of positive charges that invade the cytosol depolarize the membrane. To maintain a negative potential that facilitates CRAC channel function, an efflux

of positive charges occurs[178]. Two potassium channels have been extensively described: the voltage-gated potassium channel, shaker-related subfamily, member 3 ($K_v1.3$)[179] and the potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 ($K_{Ca}3.1$)[180].
Another channel that works precisely in the opposite direction is the transient recetor potential melastatin 4 (TRPM4). TRPM4 has been claimed to drive membrane potential to positive values, thus stopping the driving force of Ca^{2+} entry[181].

Finally, mitochondria has also been claimed to play a role on T cell activation by modulating CRAC channels. As mentioned before (see sCDI) their ability on buffering calcium is responsible for the store independent sCDI. As T cells get activated, mitochondria migrate to the IS and accumulate Ca^{2+}. Modulation of this process has been claimed to affect SOCE[182–184].

2.4. CRAC channelopathies

An effective Ca^{2+} signaling downstream CRAC channels is necessary for T cell activation. Conditions where CRAC activity is decreased or absent lead to a malfunction of the immune system.
SOCE's downstream events affect transcription factor activity and gene program activation. One of the most studied ones is NF-AT[185], but other transcription factors are affected under SOCE deficiency like activating transcription factor 2 (ATF2)[186], myeloid eIf-1-like factor (MEF)[187] and cAMP response element binding (CREB)[188].
Patients with CRAC mutations are classified under the genetic disease named "CRAC channelopathies". This disease implies

mutations on STIM1 or Orai1, giving relevance to these two proteins over their family members (STIM2, Orai2 and Orai3) as they do not compensate this malfunction. Moreover there has not yet been described any mutation on the other family members. On table 2 there is a summary for STIM1 and Orai1 mutations in human patients (reviewed in[189]).

2.4.1. STIM and Orai mouse models

Mouse studies have revealed a more detailed understanding of the *in vivo* effects of STIM and Orai proteins.

The first observation upon deletion of *stim1*, *stim2* or *orai1*, (the 3 main genes studied) demonstrated that they are needed for survival. For this reason, fetal liver chimeras or conditional mice strategies have been developed to study the functions of these proteins in the immune system. In this chapter, a main focus on T cell physiology will be described.

Orai1 gene knock out (KO) causes SOCE and cytokine production impairment in naïve and differentiated T cells. Interestingly, in these mice thymic development remains unaffected[190]. Another work has focused on the pro-survival effects on T cells after Orai1 depletion, concluding that Orai1/NF-AT axis is crucial to induce T cell death[191].

A different transgenic mouse model using nonfunctional Orai1-R93W protein showed that T and B cells display a defective SOCE, cytokine production and fail to reject a skin allograft. The authors also saw that T cells from this transgenic mice failed to induce T-cell mediated inflammatory bowel disease (IBD)[195].

Table 2

	ORAI1			STIM1	
Gene defect	R91W	A88SfsX25	A103E / L194P	E128RfsX9	1538-1 G>A
mRNA	Yes	No	Yes	No	Decreased
Protein	Yes	No	No	No	No
SOCE	No	No	No	No	No
I_{CRAC}	No	No	Nd.	Nd.	Nd.
Primary Immuno deficiency	Yes	Yes	Yes	Yes	Yes
Autoimmunity	–	Yes	–	Yes	Yes
Ectodermal dysplasia, anhidrosis	Yes	Yes	Yes	Yes	Yes
Muscular hypotonia	Yes	Yes	Yes	Yes	Yes
Ref.	125	192	192	193	194

Table 2: Mutations on ORAI1 or STIM1 that cause CRAC channelopathies. (Nd; not determined) Modified from[189].

Loss of function of *stim1* in transgenic mice has a similar phenotype as *orai1* KO. *Stim1* deficient mice present a loss of SOCE on T cells, whereas *stim2* deletion has a minor effect. Despite the mild effect on SOCE, *stim2* deficient mice were unable to induce NF-AT nuclear translocation as well as cytokine production[196]. Moreover studies in double knock out (DKO) mice for *stim1* and *stim2* showed a lymphoproliferative phenotype and autoimmune disease. This phenotype was related to a decreased regulatory T cell subset population, suggesting a crucial role for CRAC machinery in the development of this T cell subset[196].
Furthermore, it has been shown that CD4 cells lacking *stim1* were unable to produce experimental autoimmune encephalomyelitis whereas *stim2* deicient mice presented a moderate phenotype[197].

DKO studies in the same model showed the same outcome[198]. Interestingly, conditional CD4 DKO mice also develop spontaneously primary Sjorgen's Syndrome (pSS) with salivary gland immune infiltration[199].
Finally, CD8 T cells lacking *stim1* and *stim2* show inability to exert antitumoral immunity[200].

2.4.2. CRAC dysfunctions: emerging roles

SOCE is very important for a proper immune function as described above, but new emerging roles for this proteins on other systems like endothelial function[201], muscular homeostasis[202,203] and smooth muscle function are just emerging.

As described before, asthma has a strong immune system's component, but airways remodeling as well as airway smooth muscle cells hyper-responsiveness are hallmarks for this disease[204,205]. The role of CRAC channels in airway pathology has been explored under the basis of how this channel control vascular smooth muscle cells proliferation and migration. Platelet-derived growth factor (PDGF) agonist binds to vascular smooth muscle cells (VSMCs) provoking I_{CRAC} development. This, drives cell migration and proliferation (reviewed in[206]) events related to asthma disease[207].
Endothelial cells are also affected by SOCE. Orai1 and STIM1 are important for endothelial proliferation[208] and mediate cellular regeneration after vascular injury[209,210].
Thus, emerging roles for STIM-Orai axis will surely increase our understanding on cellular physiology for different systems. The observation of the extra immune symptoms that CRAC

channelopathies comprise (see table 3), highlight the relevance of this axis not only in the immune system but also on other cell types.

ii. OBJECTIVES

Objectives

The general hypothesis behind the main objectives of this thesis project comes from two observations: i) ORMDL3 gene expression is a risk factor for proinflammatory diseases. ii) the protein encoded by this gene participates in cellular calcium homeostasis. Thus, we think that ORMDL3 pathophysiological association might be mediated in part by altering the physiology of T lymphocytes, an immune cell type highly dependent on intracellular calcium signaling pathways.

Our main objective is to study the role of ORMDL3 protein in T cell physiology focusing on the activation process.

Specific objectives

1. To study the effect of ORMDL3 expression levels on T cell activation

2. To characterize ORMDL family in primary isolated T lymphocytes

3. To study the ORMDL complex integrity and composition in mammalian cells.

iii. RESULTS

CHAPTER 1

ORMDL3 modulates store-operated calcium entry and lymphocyte activation.

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CHAPTER 2

Physiological genetic modulation of *ORMDL3* expression and T lymphocyte activation

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Manuscript in preparation

Physiological genetic modulation of *ORMDL3* expression and T lymphocyte activation

Amado Carreras-Sureda, Kerstin Kiefer, Fanny Rubio-Moscardo, Miguel A. Valverde and Rubén Vicente

ABSTRACT

Several genome-wide association studies have demonstrated a relationship between *ORMDL3* and proinflammatory diseases like asthma, inflammatory bowel diseases or rheumatoid arthritis. In lymphocytes, *ORMDL3* expression levels modify calcium signaling and NFAT-mediated lymphocyte activation. However, the expression of other members of the ORMDL family in lymphocytes and their relevance to lymphocyte physiology is poorly characterized. The aim of this work was to characterize the expression profile of ORMDLs depending on the activation status and on the presence of disease-associated polymorphisms near *ORMDL3* gene. Our results showed an increased expression of ORMDL proteins under activation stimulus in mice and human lymphocytes. The main contributor to this induction in both species is ORMDL2. Besides, T cell polarization studies revealed different expression pattern depending on the Th subpopulation. Data on human T cells confirmed the relationship between *ORMDL3, GSDMB* and *ZPBP2* expression levels and the asthma-related rs7216389 single nucleotide polymorphism (SNP). TT carriers expressed higher levels of ORMDL3 than CC carriers at resting conditions, but this difference was lost following T cell activation. These changes in

ORMDL3 expression correlate with changes in calcium signaling pathway during activation. Most interestingly, our results monitoring also showed that several markers of activation (IL2, CD25 and CD69) differed between alleles, reinforcing the idea that *ORMDL3* expression affects calcium signaling and thereby lymphocyte physiology. In conclusion this work characterizes the differential expression of the whole ORMDL family in T cells and highlights the *ORMDL3* chromosomic region as a modulator of lymphocyte activation.

INTRODUCTION

ORMDL3, a member of the Orosomucoid-like proteins family (ORMDL) captured the attention of the scientific community when appeared in 2007 associated to childhood asthma in a Genome Wide Association Study (GWAS)[1]. This first association to asthma disease has been later on confirmed in different genetic population studies and extended to adult asthma and severe asthma on other GWAS[2–7]. Although the disease-associated SNPs are not present in the ORMDL3 gene's coding region, it has been shown that the alleles associated to the disease exert a positive regulation on *ORMDL3* gene transcription. Besides, this regulation in *cis* affect other genes present in the same chromosomic region like *IKZF3*, *ZPBP2*, *GSDMB*[8].

The relevance of ORMDL3 protein in asthma pathophysiology has been further confirmed in animal models. ORMDL3 expression is enhanced in lung epithelium of ovoalbumin induced asthmatic mice[9]. This upregulation would increase the production of chemokines, metalloproteases and other paracrine

factors involved in asthma disease. However, these findings have not been replicated in another work[10]. More recent studies using a transgenic ORMDL3 constitutive overexpressing mouse model have reported airways remodeling preceding immune infiltration, favoring the idea that ORMDL3 pathophysiology in the airways might come from an epithelial dysfunction[11].

SNPs in the chromosomic region surrounding *ORMDL3* gene are associated to proinflammatory diseases like immflammatory bowel disease (IBD), diabetes type I (T1D) or rheumatoid arthritis (RA)[12–18]. Other parameters involved in immune system physiology, like number of white blood cells[19] or IL-17 production[20] are also modified by the allelic pattern of SNPs in this chromosomic region. Moreover, previous work from our laboratory and others have shown that ORMDL3 modulates calcium homeostasis, activation of immune cells[21] and regulates eosinophil degranulation and trafficking[22].

Considering all the aspects that link ORMDL3 with immune system physiology, this work aims to characterize the ORMDL family in T lymphocytes in order to have a better picture of the pathophysiological role of these proteins. For this purpose, we have studied the expression levels of the different ORMDL family members during the process of activation of primary cultured mouse and human T cells. We have also analyzed the pattern of expression in different Th subsets after T cell polarization. Finally we have studied the relevance of the asthma-associated rs7216389 SNP in relation to expression and activation parameters in human T cells. Our work shows that this risk allele not only modulates *ORMDL3* expression but also determines calcium signaling and early activation parameters in human T cells.

RESULTS

Increased expression of ORMDLs during T cell activation in mice

We have studied the physiological regulation of ORMDLs during T lymphocyte activation in isolated T cells from mice. Using an antibody that recognizes all three members of the ORMDL family, we observed a time-dependent up regulation of ORMDL proteins under CD3/CD28 stimulation for 24h (Fig. 1A). Due to the absence of specific antibodies for each of the three ORMDLs, we analyzed the contribution of each member by real time PCR. Our results showed that *ORMDL2* transcription is induced during activation in a greater extent than the other isoforms with a peak at early time points (Fig. 1B). Expression analysis on activated $CD4^+$ T cells during 3 days under non polarizing conditions (ThN) confirmed the increased expression of ORMDLs up to 20 fold compared to resting cells (Fig. 1C).

Differential expression pattern of ORMDLs in mouse T cell subsets

There are several $CD4^+$ cell subsets in charge of the organization of specific immune responses depending on the insult. Any genetic modification that causes an unbalanced response of any of these subtypes might be the origin of inflammatory disease. In this sense, we wanted to study whether *ORMDL* genes presented differential regulation after T cell differentiation in order to correlate ORMDL expression profiles with T cell subpopulations' activity. Our results show that despite there are no major changes in ORMDL protein amount depending on the Th subset (Fig. 1D), ORMDL genes are highly regulated and the

specific ORMDL composition might vary during differentiation (Fig. 1E). Thus, we observed lower expression of *ORMDL1* on all T cell subsets, a strong reduction of *ORMDL2* expression in regulatory T cells (Treg) and a differential regulation of *ORMDL3* in the classical Th1/Th2 classification (Fig. 1E). We also observed that *ORMDL3* is the only member that did not reduce its transcription levels after Treg differentiation.

ORMDL and SPTLC expression during human T cell activation

The changes in ORMDLs expression were also evaluated in T cells isolated from human peripheral lymphocytes and activated for 24h with plate bound anti CD3/CD28. Our results demonstrated the induction of ORMDLs protein levels in activated T cells (Fig. 2A-B). The analysis of the individual ORMDL isoforms by real time PCR confirmed that, similarly to mice, ORMDL2 is the main contributor to the increased expression of ORMDLs by transcriptional regulation (Fig. 2C).

ORMDL3 plays a role in calcium homeostasis[23] and the whole ORMDL family mediates *de novo* sphingolipid synthesis acting as inhibitors of the serine palmitoyltransferase (SPT) complex[24]. One of the signaling pathways necessary for the correct T cell activation depends on intracellular calcium concentration increases[25]. However, little is known about the role of *de novo* sphingolipid synthesis during T cell activation. Considering the relationship between ORMDLs and sphingolipid synthesis, we studied the changes in SPT human proteins SPTLC1 and SPTLC2 following human T cell activation. Our expression studies showed an increased expression of SPTLC1 after 24h of activation with CD3/CD28, indicating that probably this

pathway is necessary for the correct functioning of T cells (Fig. 3 A-B).

The rs7216389 SNP modulates the expression of nearby genes in human T cells

It has been previously reported that common SNPs in the chromosomic region 17q12-q21 change the expression of genes within this region[8]. Therefore, we evaluated the expression of the different genes present in the proximity of the rs7216389 under resting and activated conditions. The genes studied were *ORMDL3*, *GSDMB*, *ZPBP2* and *IKZF3.* The comparison of the basal expression levels reported an increased transcription of *ORMDL3* and *GSDMB* in resting lymphocytes for TT carriers versus CC carriers in agreement with previous reports[1]. On the other hand, *ZPBP2* showed reduced expression, while *IKZF3* transcription was not affected (Fig. 4A). Interestingly, the expression differences between alleles were lost upon induction of lymphocyte activation at early (4h) and late (24h) time points and varied differently among the different genes (Fig. 4B-E). *GSDMB* showed a marked repression of the transcription following activation with *ORMDL3* following a similar trend. On the contrary, *ZPBP2* and *IKZF3* showed a clear induction during activation (Fig. 4B-E)

rs7216389 SNP-dependent modulation of human T cell activation

Our laboratory has previously shown that expression levels of ORMDL3 are inversely correlated with the Store Operated Calcium Entry (SOCE) pathway[21], a key signaling event in the activation of T cells[25]. Therefore we decided to evaluate the impact of the rs7216389 SNP on SOCE triggered by store depletion in

resting and activated lymphocytes from healthy human volunteers. Our calcium analysis showed that SOCE is reduced in homozygous carriers of the risk rs7216389 SNP (TT carriers) in resting lymphocytes compared to CC carriers (Fig. 5A) and that this difference is lost after activation for 24h with CD3/CD28 (Fig. 5B). Considering that the rs7216389 SNP increases *ORMDL3* expression at resting conditions (Fig. 4B and 4E), and the inverse correlation between ORMDL3 levels and SOCE[21] the present data supports the idea that genetically programmed increase ORMDL3 expression reduces SOCE.

Given the differences found in calcium levels between TT and CC carriers, we decided to check activation parameters in T cells from volunteers with allelic differences in SNP rs7216389. We have evaluated the expression of IL-2, CD25, CD69 and INF-gamma by real time PCR at different time points following T cell activation. Our results showed a significant reduction in IL-2, CD25 and CD69 (and a similar trend in IFN-gamma) in TT carriers, compared to CC carriers (Fig. 5C-F). These differences disappeared at 24h.

DISCUSSION

The linkage of SNPs surrounding *ORMDL3* gene to proinflammatory diseases, like asthma, ulcerative colitis, Crohn's disease or rheumatoid arthritis by Genome Wide Association Studies suggested that expression levels of *ORMDL3* might influence the physiology of the different cell types involved in these pathologies. However, compared with the amount of works exploring the association of these SNPs in different human

populations, there are few ones exploring the functional impact of ORMDLs in cell physiology. Regarding their cell function, the third member of this family of proteins modulates cellular calcium homeostasis by affecting the internal calcium stores buffering capability, specifically mitochondria and endoplasmic reticulum[21,23]. In addition, ORMDLs act as sensors of cellular ceramide content and inhibit the *de novo* sphingolipid synthesis pathway[24].

Considering the inflammatory profile shared by all the diseases genetically associated to *ORMDL3*, we decided to characterize the expression of ORMDLs in lymphocytes in order to have a better understanding of the pathophysiological role of these proteins. Our results showed that the three ORMDL members are expressed in T lymphocytes and their expression is altered following activation of these cells. Based on the combined protein and mRNA analysis, we propose that the higher amount of ORMDL protein in activated lymphocytes may come from an increased *ORMDL2* transcription. However, since there are not isoform-specific antibodies available, we cannot rule out the possibility that posttranslational regulation might occur and ORMDL1 and 3 would contribute significantly to the overall ORMDL protein increase.

There are different Th cell subpopulations whose function and dysfunction define phenotypical aspects of inflammatory diseases. Thus, asthma disease and ulcerative colitis are caused or promoted by an exacerbated Th2 response[26,27]. On the contrary, Crohn's disease is typically linked to an altered Th1, Th17 response in the gut (reviewed in[28]). Besides, impairment of Treg functioning is known to cause an exacerbated inflammatory response in most of the inflammatory diseases[29]. Therefore we

have characterized the transcriptional profile of ORMDLs in different Th subtypes in order to determine Th populations sensitive to variation in the expression of one particular ORMDL member. Our results showed reduced transcription levels of *ORMDL1* gene in polarized lymphocytes. More interesting is the regulation that occurs in Treg cells where *ORMDL2*, the main isoform induced during activation, together with *ORMDL1* are repressed whereas *ORMDL3* is the only member maintaining the expression levels. This may suggest a role for ORMDL3 in the immunosuppressive Treg subpopulation. It is also remarkable the unexpected lower gene expression in Th2 subtype of *ORMDL3* since Th2 like environments are claimed to induce *ORMDL3* expression[9,22,30] . Whether this regulation is conserved in humans and most importantly, the possibility that common polymorphisms present in the general population may alter T cell differentiation, are important questions that need further work.

We have studied the expression profile of ORMDLs depending on the risk allele present in SNP rs7216389. Our results confirmed what has been previously published by other authors; TT carriers show a higher transcription level than CC carriers[1]. These differences, however, are lost during the activation process, suggesting that other elements in the promoter, induced by the activation cascade, acquire dominancy. In addition, we have confirmed that SNP rs7216389 modulates other genes located in the same chromosomic region. Thus, TT carriers have increased *GSDMB* expression but decreased *ZPBP2* expression. On the contrary, *IKZF3* remains insensitive to this *cis* regulation. Similar gene expression regulation was observed for another SNP in the chromosomic 17q12-21 region, rs12936231[8]. Interestingly, the

transcriptional activation program abolishes allelic differences and affects differentially all these genes supporting idea that, upon activation, a new scenario of transcriptional factors takes control of the gene expression in this region.

Furthermore, we have demonstrated that there are differences in T cell early activation markers depending on the rs7216389 SNP. Thus, the fact the basal changes in the expression of ORMDL3 might be modulating functional aspects of lymphocyte physiology would provide the first straight connection between genetic studies and pathophysiology of this gene. In this sense, we have observed that store operated calcium entry in resting T cells is lower in TT carriers in agreement with our previous studies showing that ORMDL3 reduces SOCE in Jurkat T cells[21]. We think that the lower SOCE might explain the alterations in early activation. Nevertheless, we cannot discard the impact of other genes whose expression is also influenced by the SNP rs7216389, *GSDMB* and *ZPBP2,* in the activation process.

ORMDLs are proteins regulating two main functions of the endoplasmic reticulum: lipid synthesis and calcium homeostasis. However, the mechanism linking these proteins to proinflammatory diseases is not fully understood. The ORMDL3-mediated reduction in SOCE and other markers of T cell activation (Fig. 5) fits better with an immunosuppressive rather than with a proinflammatory role, thereby in apparent contradiction to the data implicating ORMDL3 in T cell dependent inflammatory diseases. However, two critical aspects should be taking into account in order to discuss the role of ORMDL3 in inflammatory diseases. First, the risk allele TT for asthma is the protective one for diseases like type 1 diabetes, Crohn's disease and primary biliary chirrosis[8]. Second,

the influence of ORMDL3 expression in Treg cells could be critical to understand its involvement in pathophysiology since this population is sensitive to SOCE alterations and this might explain the inflammatory and autoimmune phenotypes[31]. We cannot discard that SPT activity might also differ depending on the SNP given the differences in ORMDL3 expression at basal levels. In this respect, we cannot link the induction of ORMDL protein as an inhibition in *de novo* sphingolipid synthesis during activation since SPTLC1 is also induced.

In conclusion, this work demonstrates that ORMDLs expression is tightly regulated during T lymphocyte activation and differentiation. Moreover, this regulation is influenced by polymorphisms in the chromosomic region 17q12-q21, associated to proinflammatory diseases. Finally, we show for the first time that common genetic variants modify calcium signaling and activation of circulating human T cells. Whether these differences are related to the pathophysiology of ORMDL3 is an attractive idea that would need further work to demonstrate.

MATERIALS AND METHODS

T cell isolation and differentiation.

Mouse CD4+ and CD8+ or only CD4 T cells were isolated from lymph nodes and spleens of C57BL/6 wild type mice by Dynal magnetic bead separation (Life sciences) according to the manufacturer's instructions. CD4 cells were differentiated to ThN, Th1, Th2, Th17 and Treg with plate-bound anti-CD3 and anti-CD28 Abs for 2 days, followed by culture in RPMI medium

containing either IL-2 alone (ThN, non polarizing conditions); IL-2 plus IL-12 and anti-IL-4 (Th1 conditions); IL-2 plus IL-4 (Th2 conditions); IL-2 plus TGFβ (Treg conditions); IL-2 plus IL-6, TGFβ, anti-IL-4 and anti IFN-γ (Th17 conditions). Human T cells were isolated from PBMCs describe in full by enhanced human T-Cell immunocolumns (Diagnóstica Longwood, S.L.) and cultured with plate-bound anti-CD3 and anti-CD28 (BD biosciences).

Protein analysis

For western blotting total protein lysate was separated on 4–12% gradient polyacrylamide gel and transferred to PVDF membranes. Primary antibodies used were: mouse anti-ß-Actin (1:5000), rabbit anti-ORMDL (1:300), rabbit SPTLC1 (1:300) and rabbit SPTLC2 (1:1000) all from Abcam. Secondary antibodies were horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (1:3000, GE Healthcare). Immunoreactive signal was detected by SuperSignal West Chemiluminiscent substrate (Pierce) and visualized by Molecular Imager Chemidoc XRS system (Biorad).

Real time PCR analysis

Extraction of total RNA from human or mice T cells was performed following manufacturer's instructions (Nucleospin RNA II kit, Macherey-Nagel). cDNA was obtained using SuperScrip-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). For human samples, ORMDL1, ORMDL2 and ORMDL3 primers were obtained from QuantiTect Primer Assay (Qiagen). Other human primers used included: hIL-2 5'-AACTCACCAGGATGCTCACA-3

and 5'-GCACTTCCTCCAGAGGTTTG-3'; hCD25 5'-CCTGGGACAACCAATGTCA-3' and 5'-TGGACTTTGCATTTCTGTGG-3'; hCD69 5'-TCTTTGCATCCGGAGAGTG-3' and 5'-GCACACAGGACAGGAACTTG-3'; MLN5 5'-ACCAGACCGGCCACCAT-3' and 5'-CAAGGAAGGTCGTGCTGGTT-3'; hIKZF3 5'-TGGAAAATGTGGACAGTGGA-3' and 5'-CATTTCCCATGGGTTCTGAC-3'; hZPBP2 5'-CTGGACAGCTGATGGTGAAA-3' and 5'-CCCGATAGGCAAAGACCATA-3'; hGSDMB 5'-ACATGGAGGACCCAGACAAG-3' and 5'-CACAGAGAATTCGTGCCTCA-3'; hIFNgamma 5'-TGACCAGAGCATCCAAAAGA -3' and 5'-CTCTTCGACCTCGAAACAGC -3'. Mouse primers used: mORMDL1 5'- GCATCCCCTTCTGCAGTGTT-3' and 5'-CGGAGTCTCAAAAGGCGTTC-3'; mORMDL2 5'-CGTCATCCATAACTTGGCAAT-3' and 5'-AACTGTAGTCCATAGTCCATC-3'; mORMDL3 5'-CTGCTGAGCATTCCCTTTGT-3' and 5'-CACGGTGTGCAGAAAGATGT-3'; mL32 5'-ACCAGTCAGACCGATATGTG-3' and 5'-ATTGTGGACCAGGAACTTGC-3'. SPTLC1 and SPTLC2 primers were described previously[32] . IKZF3, GSBB and ZPBP2 were previously described in[8]. All primers are noted in forward and reverse, respectively. PCR conditions for all cases were: 95°C for 5 min; 95°C for 30 s; 60° for 30 s, 72°C for 30 s; 72°C for 5 min; with 40 cycles of amplification.

Human T cell sample genotyping

Human DNA was extracted using white cell lysis buffer containing: 100 mM Tris-Cl (pH 7.6), 40 mM EDTA (pH 8.0), 50 mM NaCl, 0.2% SDS and 0.05% Sodium azide. After lysis a salt solution (6M NaCl) and ethanol precipitation protocol was used to purify DNA. Genotypation of SNP rs7216389 was done by amplifying the surrounding region using the following primers 5'-GTGCCTGGCATACATTCTAACTGC-3'and 5'-AGCCCTGCCTCCAAAACCTAG-3 with Biotaq DNA polymerase (Bioline). Cycle sequencing was performed on purified PCR products with Applied Biosystems BigDye terminator v3.1 sequencing chemistry and run on an ABI 3100 (Applied Biosystems, California, USA) genetic analyzer. The sequences were analyzed with DNAstar Lasergene 11 software.

Calcium imaging

Cytosolic Ca^{2+} signal was determined in cells loaded with 4,5 μM fura-2 AM (20 min) as previously described[21]. Cytosolic [Ca^{2+}] increases are presented as the ratio of emitted fluorescence (510 nm) after excitation at 340 and 380 nm, relative to the ratio measured prior to cell stimulation (fura-2 ratio 340/380). All experiments were carried out at room temperature and cells were bathed in a solution containing (in mM): 140 NaCl, 5 KCl, 1.2 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, 10 HEPES (300 mosmol/l, pH 7.4 with Tris). Ca^{2+}-free solutions were obtained by replacing $CaCl_2$ with equal amount of $MgCl_2$ plus 0.5 mM EGTA. Stores depletion was achieved in human T cells using 30 μM cyclopiazonic acid (CPA).

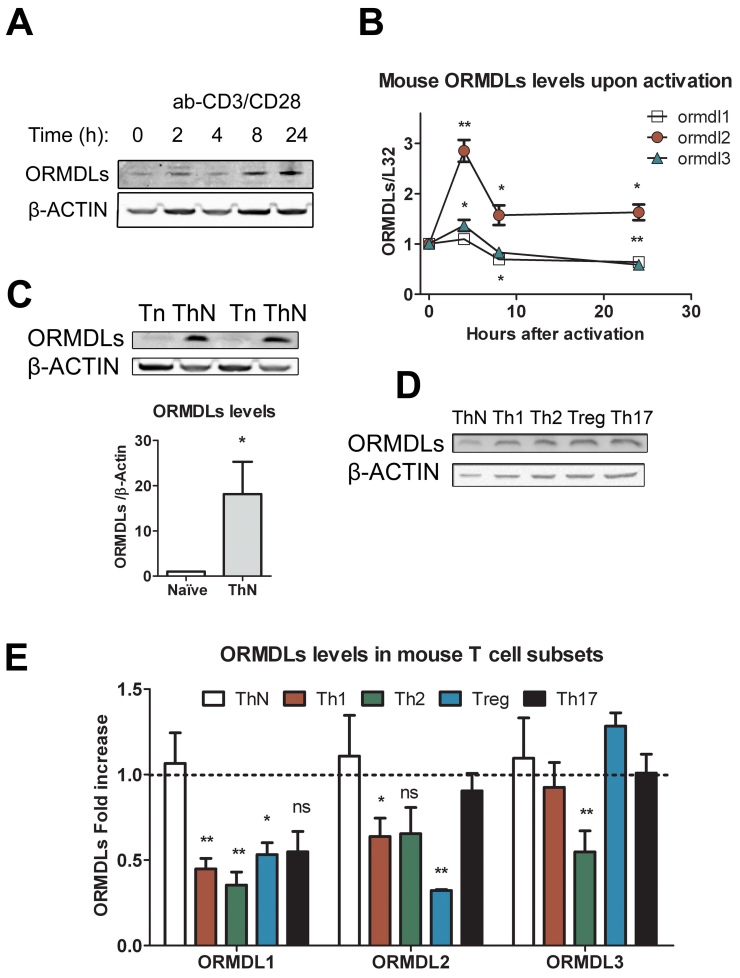

Figure 1: ORMDLs are regulated upon T cell activation. **A**. Representative WB of mouse T cells stimulated with plate bound-CD3/CD28 antibodies at the indicated time points. **B**. Real time studies on mouse CD3/CD28 activated T cells for ormdl1, ormdl2 and ormdl3 transcripts (n=4). **C**. WB of mouse T cells polarized to ThN blotted against ORMDLs (n=4). **D**. Representative WB of mouse T cells polarization. **E**. ORMDLs expression pattern in different polarized T cells (n=6). **p<0.01; *p<0.05.

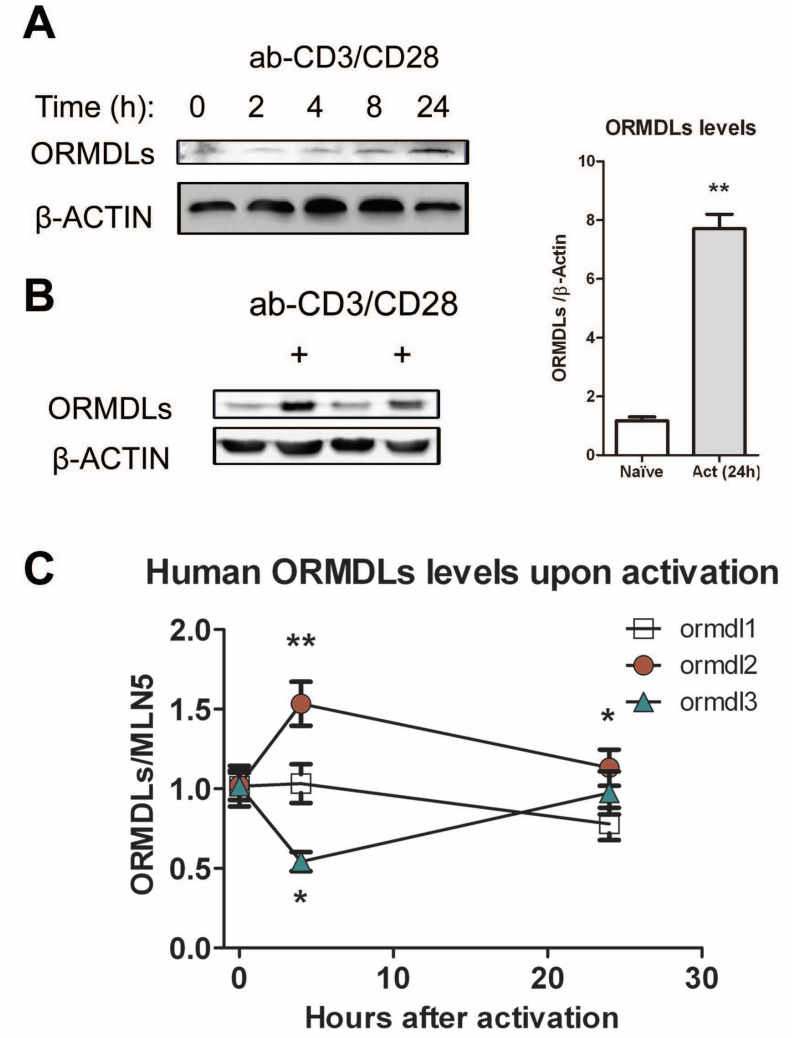

Figure 2: ORMDL expression during human T cell activation. **A**. Representative WB of human T cells stimulated with plate bound-CD3/CD28 antibodies at the indicated time points. **B**. WB of human T cells stimulated with plate bound-CD3/CD28 antibodies for 24 hours and blotted against ORMDLs (n=4). **C**. Real time studies on human CD3/CD28 activated T cells for ORMDL1, ORMDL2 and ORMDL3 transcripts (n=6).

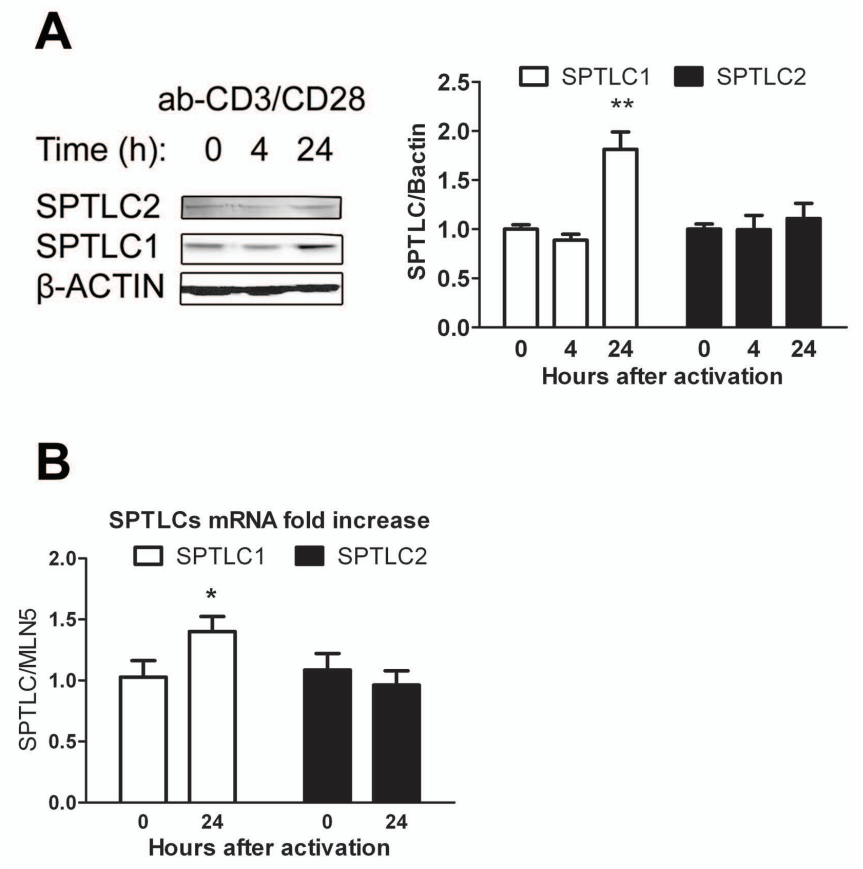

Figure 3: SPTLCs are regulated under T cell activation. A. SPTLC1 and SPTLC2 expression levels by WB at resting, 4 and 24 hours after stimulation of human T cells with CD3/CD28 (n=3). **B**. SPTLC1 and SPTLC2 expression levels by real time PCR of resting and 24 hours stimulated human T cells with CD3/CD28 (n=4).

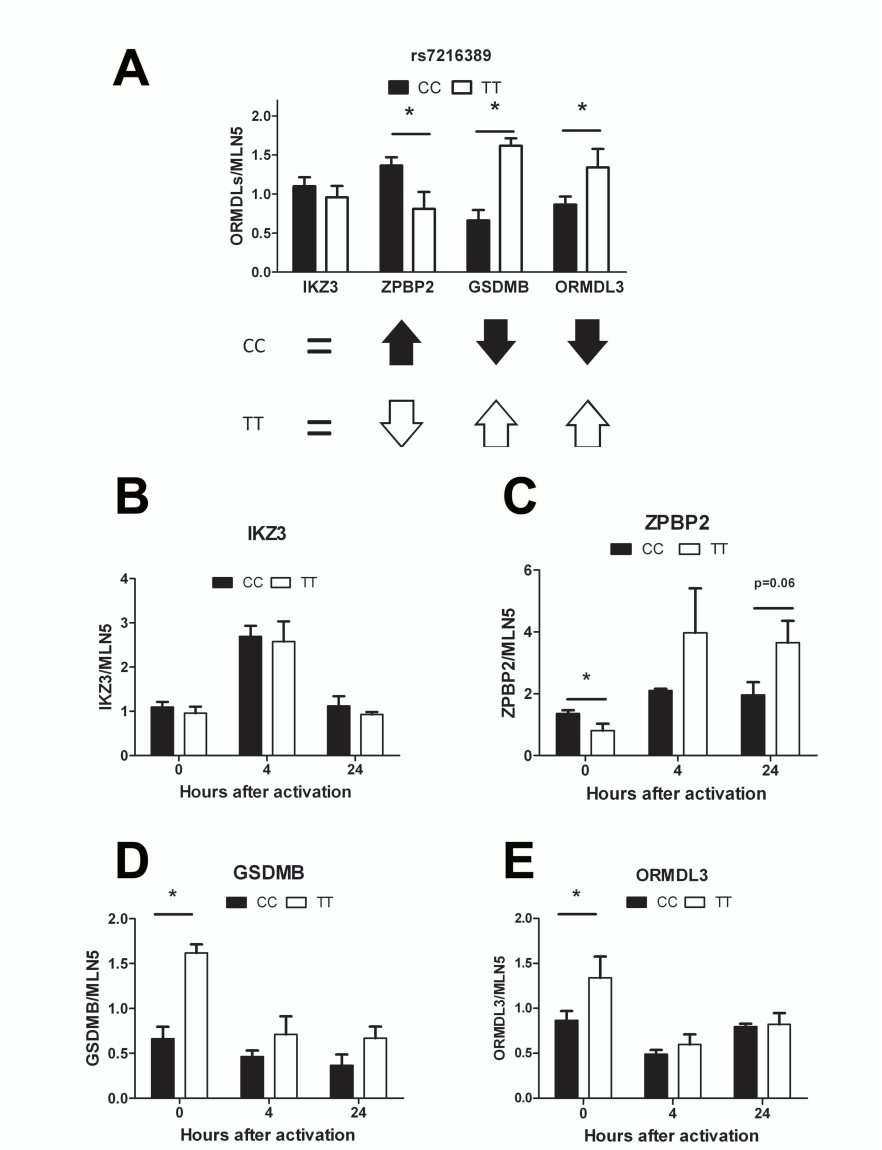

Figure 4: SNP rs7216389 determines surrounding genes expression. Expression levels of *IKZ3F*, *ZPBP2*, *GSDMB* and *ORMDL3* on human resting T cells (**A**) or CD3/CD28 activated T cells at the indicated time points (**B-E**) classified based on SNP rs7216389 (n= 8-11). **p<0.01; *p<0.05.

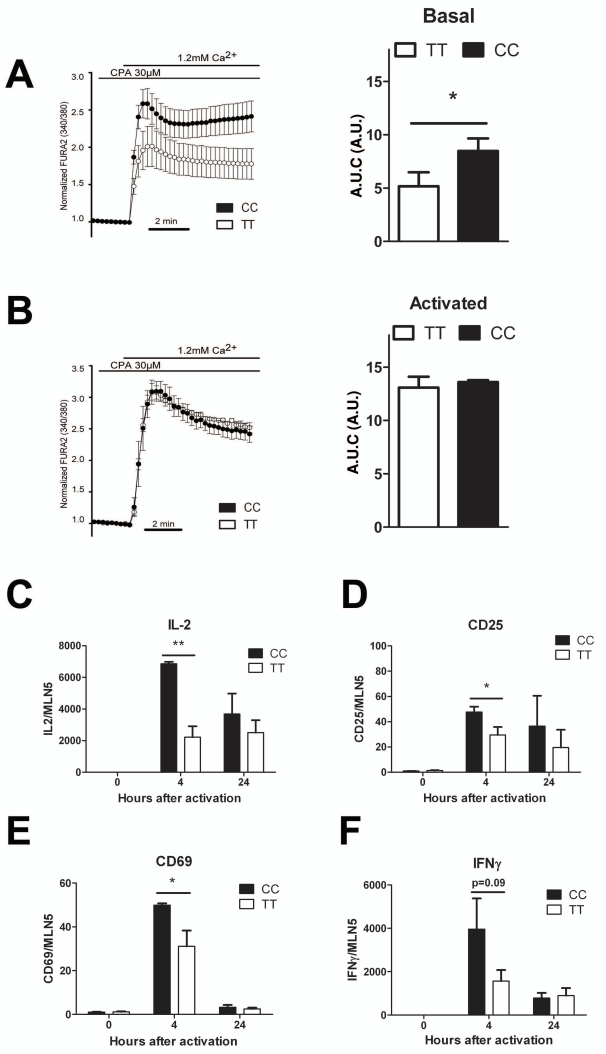

Figure 5: SNP dependent kinetics during T cell activation. A. Store operated calcium entry (SOCE) on human T cells after store depletion with CPA on resting cells or (**B**) after 24 hours of CD3/CD28 treatment. Y axis represents the Area Under the Curve (A.U.C.) in Arbitrary Units (A.U.); (n=6-10). **C-F**. Real time studies at resting, 4h and 24 hours of stimulated human T cells with CD3/CD28. **C**. IL2 expression. **D**. CD69 expression. **E**. CD25 expression. **F**. IFN-γ expression. (n=6-8). **p<0.01; *p<0.05.

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CHAPTER 3

ORMDL family complex rearranges under different lipid environments

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ABSTRACT

Orosomucoid like proteins (ORMDLs) are endogenous inhibitors of the serine palmitoyl transferase (SPT), the first enzyme in the *de novo* sphingolipid synthesis pathway. ORMDLs are transmembrane proteins of the endoplasmic reticulum that oligomerize and interact with SPT depending of the lipidic environment. The mechanism of this regulation has been studied mainly in yeast where there are two orthologs of the family called Orms. In this system, the formation of the complex SPT-Orms is regulated by phosphorylation of the N terminus of Orms downstream of cellular stress pathways. However, this regulatory domain is lost in mammalian isoforms and little is known regarding mammalian regulation. In the present work we study the interaction between mammalian ORMDL isoforms and SPT. By using a combination of co-immunoprecipitation assays and fluorescence resonance energy transfer (FRET) experiments, we demonstrate that, similar to yeast Orms, mammalian ORMDLs form an oligomeric complex that binds to SPT human isoforms (SPTLC1 and SPTLC2). Interestingly, and contrary to the mechanism described in yeast this interaction is insensitive to changes in the lipid environment, suggesting a mechanism of SPT inhibition by ORMDLs different to the one reported in yeast. Nevertheless, we do observe that proximity of C tails varies depending on the cellular sphingolipid composition, evidencing a lipid-dependent internal

rearrangement in the complex. Therefore, we picture a model where ORMDLs form hetero-oligomers between themselves and with SPTLCs that may rearrange internally, without losing their interaction, depending on the lipid environment.

INTRODUCTION

Ceramides and sphingolipids are intracellular regulators of cell fate as they can mediate different processes like inflammation, angiogenesis or apoptosis[1,2]. Ceramides can be produced from degradation of complex lipids or synthetized *de novo* via the sphingolipid synthesis pathway at the endoplasmic reticulum (ER). The serine palmitoyltransferase (SPT), that catalyzes the condensation of a serine and a palmitoyl-CoA to form 3-Keto-sphinganine, is the first rate limiting enzyme for *de novo* sphingolipid synthesis[3,4]. SPT complex is composed by a dimeric structure formed by SPT long chain 1 (SPTLC1) with either SPTLC2 or SPTLC3[5]. In 2010 a screening on genes involved in sphingolipid homeostasis in yeast revealed that Orm1 and Orm2, yeast orthologs of ORMDLs, bind to and control SPT activity[6]. The mechanism behind the Orm-mediated regulation of sphingolipid synthesis involves the oligomerization of Orm1 and Orm2, a process controlled by phosphorylation of the N terminus of these proteins. This phosphorylation decreases Orm binding to the SPT complex and the subsequent release of its inhibitory activity[6,7]. There are 2 kinases responsible for Orms phosphorylation, YPK, that in turn is controlled by TORC2[8] and Npr1 downstream TORC1[9]. Dephosphorylation is regulated on one hand by Tap42 phosphatase complex, downstream TORC1[10] and on the other hand by Cdc45-PP2A under heat stress response[11]. These pathways adapt lipid production to cell lipid demand. Thus, under

low sphingolipid environments Orm1/Orm2 are phosphorylated and present less interaction between themselves and to SPT allowing the induction of the *de novo* synthesis pathway.

ORMDL is a family of transmembrane proteins located in the endoplasmic reticulum composed by three members[12]. This family participates in the intracellular calcium and sphingolipid homeostasis. ORMDL3 the third member of ORMDL family, influences calcium content within the ER[13] and mitochondrium as well as store-operated calcium entry (SOCE) following depletion of intracellular stores[14]. These actions are dependent on the cytosolic exposed C and N terminal tails, respectively. On the other hand ORMDLs are claimed to have redundant effects regarding the inhibition of SPTLCs[15]. However, no study has addressed yet how these three isoforms interact among themselves and with SPTLCs. Interestingly, the N-terminal domain, target of phosphorylation and responsible for the regulation of the yeast orthologs Orm1/Orm2, is missing in mammalian ORMDLs, suggesting that the mechanism of regulation is not conserved. The aim of this study is to elucidate the interaction of ORMDL family members within themselves under different lipid environments as well as to evaluate the binding of this complex to SPTLC1 and SPTLC2.

RESULTS & DISCUSSON

ORMDLs form a complex

In order to evaluate ORMDL1, ORMDL2 and ORMDL3 interactions we used immunoprecipitation assays. We co-transfected HEK293 cells with myc-ORMDL3 and YFP-ORMDL1 or YFP-ORMDL2 to check hetero-oligomerization or myc-ORMDL3

with YFP-ORMDL3 for homo-oligomer formation. In all conditions tested, cells were also co-transfected with equal amounts of the other members of the ORMDL family lacking any tag. This approach would minimize artificial interactions due to the overexpression approach. Co-immunoprecipitation was checked blotting with anti-ORMDL antibody at the expected size for YFP-ORMDL constructs (42 kDa). Our results showed that, in agreement with what was observed in yeast, mammalian ORMDLs are able to interact between themselves forming oligomeric structures. This interaction showed no preferences between homomeric (ORMDL3-ORMDL3) or heteromeric complexes (ORMDL1-ORMDL3, ORMDL2-ORMDL3), varying the fraction of co-immunoprecipitation around 10% in all cases (Fig. 1A). Besides, it was previously shown that ORMDL3 is able to co-immunoprecipitate with SPTCL1 subunit in transfected HeLa cells[6]. In our experiments we further confirmed this interaction and we also observed co-immunoprecipitation of the second subunit of the SPT complex, SPTCL2. The fraction of co-immunoprecipitation was around 4% in both cases. This experiment demonstrates the existence of the ORMDL complex in conjunction with SPTLC1 and SPTLC2.

We decided to confirm the interactions observed in our pull down assays with a different approach. Thus, we tagged the C-terminus of ORMDL proteins with CFP/YFP FRET pairs in order to study interactions of the tails between different subunits of the complex. Cells were transfected not only with the YFP/CFP-tagged ORMDLs but also with the other non-tagged ORMDL members to prevent forced combinations. Our results showed positive FRET signal compared to the globular control on all the different combinations tested. Homomerization studies reveal a similar

FRET on all conditions tested (ORMDL1-ORMDL1; ORMDL2-ORMDL2 and ORMDL3-ORMDL3) (Fig. 1B). Interaction between the different members is also higher than the control condition, despite FRET signal in the ORMDL1-ORMDL2 and ORMDL3-ORMDL2 interaction is lower compared to ORMDL1-ORMDL3 (Fig. 1C). Taking into consideration that we did not observe changes in the co-immunoprecipitation studies between homomeric and heteromeric interactions, we interpreted the lower FRET signal obtained in ORMDL2 containing heteromers in terms of a different conformation of the C terminus of ORMDL2, which may be more distant to ORMDL1 and ORMDL3.

ORMDLs rearrange under different sphingolipid environments

It has been shown that the complex formed by yeast ORMDL orthologs is influenced by the amount of cellular sphingolipid composition. A decrease in the sphingolipid content reduces Orms interaction between themselves but also decreases the Orm-SPT binding[6]. Disattachment of Orm stops the brake imposed on SPT and permits the generation of new sphingolipids. Therefore, we have studied the mammalian ORMDL-SPTLC complex in a low sphingolipid environment (50% $\pm$10 reduction on total ceramide levels) by treating the cells during 4h with myriocin at 10μM, a specific inhibitor of the SPTLCs. This was compared to a rich sphingolipid environment treating the cells 4h with C6-ceramide 10μM, a permeable sphingolipid that has been shown to modulate SPTLCs in a ORMDL dependent manner[15]. Our co-immunoprecipitation experiments with myc-ORMDL3 revealed no difference neither on the ORMDL3-ORMDL3 interaction nor in the binding to SPTLC1 and SPTLC2 under low lipid environments (Myr

treatment) (Fig. 2A). Similarly, treatment with C6-Ceramide, a control for high sphingolipid loading, did not affect the integrity of the ORMDL complex nor the binding to SPTLCs (Fig. 2A). These results suggest a different behavior between Orms and ORMDLs regarding the formation of oligomeric complexes in response to changes in cellular lipid load.

Our data demonstrate that the regulation produced on SPTLC activity by ORMDLs does not involve forming or dissolving the ORMDL-SPTLC complexes. However, in order to fully understand if the lipid environment may change the structure of this complex we performed FRET experiments similar to those described above. We observed that oligomers are indeed sensitive to the lipid environment presenting higher FRET efficiencies under low sphingolipid content (Myriocin treatment) compared to high sphingolipid content (C6-ceramide treatment) (Fig. 2B). These in conjunction to our co-immunoprecipitiation experiments would suggest a rearrangement of the ORMDL complex depending on the lipid status, rather than a disattachment from SPTLC.

Together, our results suggest that, similar to Orms, ORMDLs complexes with SPTLC1 and SPTLC2. However, unlike Orms[6], we do not observe the reported decreased interaction between the complex components in response to changes in the cell lipid environment induced by myriocin and C6-ceramide. These discrepancies could be explained by the fact that ORMDLs lack the N terminal regulatory domain present in Orm1/Orm2 and responsible for the regulation of the interaction[6].

The rearrangement occurring in the complex might be related to *de novo* sphingolipid production since it is sensible to lipid environments. In this context, we propose a model where

cytosolic C tails would adopt different conformations depending on the lipidic demand with potential effects on SPTLC function (Fig. 3).

Finally, it is worth to mention that the C terminal of ORMDLs is necessary for SERCA inhibition[13]. Whether the rearrangement observed in this work influence ORMDL calcium regulation is an attractive possibility that would need further study because it would demonstrate a crosstalk of SERCA function and *de novo* sphingolipid synthesis. In this sense, it is important to mention that ORMDL3[16–22], SERCA[23], and SPTLC1[24] dysfunction have been associated to airway diseases.

MATERIALS & METHODS

Cell culture and reagents

HEK293 cells were cultured in high glucose DMEM containing 10% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin and maintained at 37 °C and 5% CO_2. HEK293 cells were transiently transfected with ExGen500 (Fermentas MBI) following manufacturer's instructions. Construct generation is described in[13]. Myriocin (10µM) or C6-Ceramide (10µM), were both obtained from Sigma and dimethyl sulfoxide (DMSO) was used as vehicle. All treatments were for 4 hours at 37 °C and 5% CO_2.

Fluorescence Resonance Energy Transfer experiments

Proximity of the different tails of ORMDLs was assessed using FRET technique. Cells were seeded in 25mm glass coverslips and transfected using the indicated combinations. In addition to the YFP/CFP pair of study in each case the rest of members were

transfected without tag in order to avoid forced interactions. Acceptor photobleaching was carried by gradual bleaching of the YFP acceptor on living cells using a SP2 confocal Leica microscope. FRET value was expressed as maximal CFP fluorescence increase compared to the initial value after the bleaching of more than 90% of YFP fluorescence signal. Images were analyzed using ImageJ software.

Immunoprecipitations assays

HEK293 cells were transiently transfected with human ORMDL3-myc plus ORMDL1, ORMDL2 or ORMDL3 with different tags as indicated. All conditions tested were also transfected with a non-tagged ORMDL protein to ensure the three proteins were present on all experiments and at the same ratio. After 24 hours of transfection, cells were lysated with immunoprecipitation buffer (0.2% Triton plus protease inhibitor cocktail in HBS) and centrifuged at 100 000g to collect total protein in supernatant. Then 1000 µg total protein was incubated at 4ºC overnight with anti-myc antibody (Abcam) cross-linked with DSS (Pierce) to Protein G sepharose beads. Immunocomplexes were washed with HBS buffer five times and eluted with Glycine 0.2M pH2.5, before adding loading buffer and boiled for 5min. Co-immunoprecipitation of ORMDL3-YFP, ORMDL1-YFP or ORMDL2 YFP was detected with by western blot separated on 4–12% gradient polyacrylamide gel and transferred to PVDF membranes. Immunodetection was carried out using anti-rabbit ORMDL (1:300), rabbit SPTLC1 (1:300) and rabbit SPTLC2 (1:1000) all from Abcam. Secondary antibodies were horseradish peroxidase-conjugated and anti-rabbit IgG (1:3000, GE Healthcare). Immunoreactive signal was detected

by SuperSignal West Chemiluminiscent substrate (Pierce) and visualized by Molecular Imager Chemidoc XRS system (Biorad).

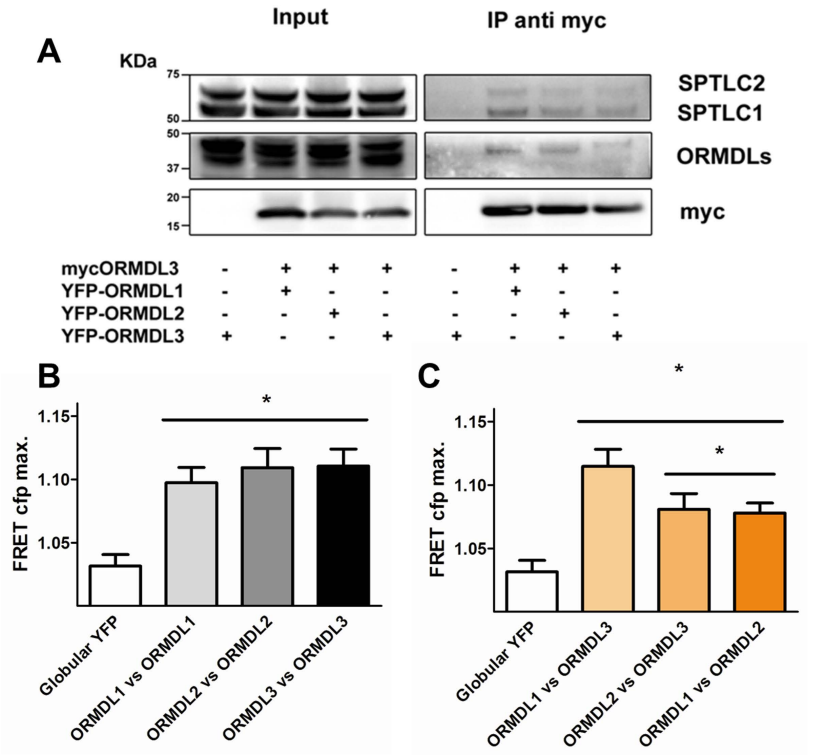

Figure 1: ORMDLs form a complex with SPT: HEK293 cells were transfected with ORMDL1 or ORMDL2 or ORMDL1+ORMDL2 plus the indicated tagged constructs to ensure every condition had the 3 members of the family. **A**. Western blot analysis of immunoprecipitations with an anti-myc antibody of different conditions as indicated. **B-C**. FRET Acceptor photobleaching studies between homomeric pairs ORMDL1 *vs* ORMDL1 (ORMDL1-CFP + ORMDL1-YFP); ORMDL2 *vs* ORMDL2 (ORMDL2-CFP + ORMDL2-YFP); ORMDL3 *vs* ORMDL3 (ORMDL3-CFP + ORMDL3-YFP); and heteromeric pairs: ORMDL1 *vs* ORMDL3 (ORMDL1-CFP + ORMDL3-YFP); ORMDL2 *vs* ORMDL3 (ORMDL2-CFP + ORMDL3-YFP); ORMDL1 *vs* ORMDL2 (ORMDL2-CFP + ORMDL1-YFP). Globular YFP (ORMDL3-CFP + PCDNA3-YFP). These data comes from 3 independent experiments (n=24). (*p<0.05)

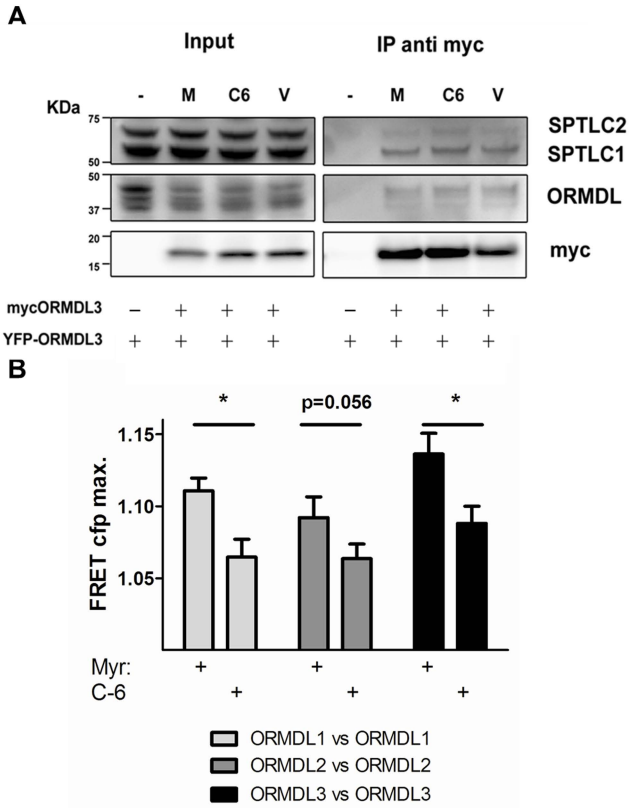

Figure 2: ORMDLs rearrange under different sphingolipid environments: HEK293 cells were transfected with ORMDL1 or ORMDL2 or ORMDL1+ORMDL2 plus the indicated tagged constructs to ensure every condition had the 3 members of the family. Myriocin (M), C6-Ceramide (C6) or DMSO (V) were applied to cells for 4 hours **A**. Western blot analysis of immunoprecipitions with an anti-myc antibody of different conditions as indicated. **B**. FRET Acceptor photobleaching studies between ORMDL members: pairs ORMDL1 *vs* ORMDL1 (ORMDL1-CFP + ORMDL1-YFP); ORMDL2 *vs* ORMDL2 (ORMDL2-CFP + ORMDL2-YFP); ORMDL3 *vs* ORMDL3 (ORMDL3-CFP + ORMDL3-YFP). Globular YFP (ORMDL3-CFP + PCDNA3-YFP). These data comes from 3 independent experiments (n=24). (*p<0.05)

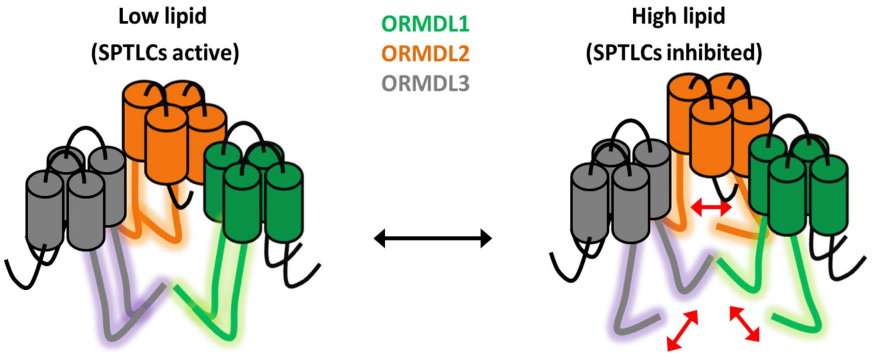

Figure 3: Model for ORMDLs' complex behavior under different lipid loading.

Oligomerization studies revealed that ORMDLs bind to each other where ORMDL2's C terminal tails would be more distant to ORMDL1 and ORMDL3. Moreover, under low sphingolipid levels the C terminal tails between the same members are in close proximity whereas lipid loading with C6 ceramide promotes a rearrangement and distancing of the C terminal tails. SPTLCs are excluded from the illustration despite they are binding the complex independently of the lipid load. Color code: ORMDL1 is green, ORMDL2 orange, and ORMDL3 grey. C terminal tails are highlighted.

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iv. DISCUSSION

Discussion

GWAS are powerful tools to determine genetic risk factors for a trait or a disease. Using these techniques many new genes have been linked to participate in pathological processes[71]. In this regard, the starting point of the work presented in this thesis was the genetic linkage between asthma disease and a gene with unknown function called *ORMDL3*. A GWAS screening for childhood asthma found one SNP in an intronic zone of the gene *GSDMB* that correlated with increased transcript levels of *ORMDL3*. This suggested that ORMDL3 expression levels were related to the risk to develop asthma [4]. After this work many others confirmed this association, but one captured our attention by providing an overview of how the chromosomic region, where ORMDL3 is located, was genetically linked, not only with asthma, but with inflammatory and autoimmune diseases. That initial work related ORMDL3 and Crohn's disease, diabetes type I or primary biliary cirrhosis[211]. On the other hand, one of the main players of the immune system are T lymphocytes. Certain exacerbated responses of these cells are related to asthma disease[212,213] and to autoimmunity[214,215]. Therefore, considering the association of ORMDL3 with inflammatory/autoimmune disease, the participation of ORMDL3 in calcium homeostasis[7], and the key role of calcium signals in T cell activation[123] we decided to explore a possible role for ORMDL3 in T cell physiology.

In this section I will try to integrate the results of this thesis and discuss them from three points of view: the role of ORMDL3 in cell physiology; ORMDL3 role in the immune system and, the pathophysiological implications of *ORMDL3* as well as other genes present in the region 17q12-21.

1. Function of ORMDL3

ORMDL3 has been involved in three main cellular events; Ca^{2+} homeostasis, control of *de novo* sphingolipid synthesis, and UPR. However, to date we do not know if these three ORMDL3 functions are consequence one of the other or they are just parallel events.

1.1. Calcium handling

The participation of ORMDL3 in the control of Ca^{2+} homeostasis was the first function attributed to this protein. This was done in our group in 2010 where we demonstrated that SERCA2b activity was inhibited by ORMDL3. The C terminal tail of ORMDL3 was identified as the domain responsible for this inhibition[7]. Interestingly, two works from a different group claimed that SERCA is up-regulated upon overexpression of ORMDL3[6,9]. To our understanding this might be a compensatory mechanism.

The work done in this thesis has increased the knowledge regarding ORMDL3 and calcium homeostasis. We have described in a model of human T cells that the N terminal part of ORMDL3 promotes a decreased mitochondrial Ca^{2+} uptake. This causes a decreased SOCE and I_{CRAC}, what is translated into less NF-AT shuttle to the nucleus and decreased IL2 production[216]. Moreover we have explored this event in human T cells and we have found that increased levels of ORMDL3 correlate with decreased SOCE and decreased early activation markers. Altogether these results point towards a role of ORMDL3 in controlling T cell activation via Ca^{2+} homeostasis. However, the mechanism underlying ORMDL3's effect on mitochondria physiology is not fully understood. We do know that this is a new function of ORMDL3 different from SERCA

inhibition or SPTLCs blockade. In this respect, we showed that ORMDL3 promoted an effect on SOCE and I_{CRAC} under conditions where we had blocked SERCA by using thapsigargin or siRNAs. Moreover, this effect was also independent of de novo sphingolipid synthesis since blockade of SPTLCs with myriocin had no effects on I_{CRAC} currents. Altogether, these results would argue that the effect on mitochondrial calcium handling is not a direct consequence of the inhibition of SERCA or SPTLC but we cannot discard completely this idea since the pharmacological approach might not fully mimic the effect of ORMDL3 on these two enzymes.

We have immunolocalized ORMDL3 at ER-mitochondria contact sites named mitochondrial associated membranes (MAMs). It is known that MAMs are very important for Ca^{2+} transfer from the ER to mitochondria in order to maintain cellular energetic homeostasis[217–219]. Different mechanisms regulate Ca^{2+} homeostasis in mitochondria: uptake mediated by the mitochondrial Ca^{2+} uniporter complex (MCUC); buffering of the mitochondrial matrix, and extrusion mechanisms, mediated by mitochondrial NCX and the proton/calcium exchanger HCX (reviewed in [220]). ORMDL3 might be affecting the calcium pathway connecting ER and mitochondria through IP_3R, VDAC and MCUC. Moreover, Ca^{2+} import into mitochondria has an intrinsic effect on this organelle. Thus, it is known that Ca^{2+} promotes ATP production and mitochondrial respiration[221,222]. However, if this uptake exceeds a certain threshold cells undergo apoptosis[223–225].
Defining the exact mechanism of how ORMDL3 controls SERCA pumps and Ca^{2+} uptake into mitochondria will increase our knowledge of the molecular events that are behind ORMDL3-associated pathologies.

1.2. Inhibition of *de novo* sphingolipid synthesis

During the first year of this thesis project two works revealed that ORMDL yeast orthologs, Orm1 and Orm2, were blocking serine palmitoyltransferase enzyme to control *de novo* sphingolipid synthesis[45,46]. On the following years the whole pathway was described. Orm1 and Orm2 inhibition of SPT requires a direct binding/interaction between these proteins, a process that is controlled by phosphorylation as well as by Orms expression levels to maintain an adequate sphingolipid content[47–51]. Moreover the first work proposed a mechanism where SPT activity relayed on the binding to Orms depending on the sphingolipid environment[45]. Mammalian ORMDLs have also been shown to interact and exert this inhibition on SPTLCs[45,52]. Therefore, we aimed to evaluate if the regulation of the complex between mammalian ORMDLs and SPTLCs followed a similar mechanism than the one described in yeast.

We have seen that ORMDLs form a heterogeneous complex that includes SPTLC1 and SPTLC2. However, this ORMDL-SPTLC complex is not affected by changes in the ceramide content of the cell, suggesting that the mechanism of inhibition on SPTLCs is not conserved from yeast to mammals. Our studies propose a model where the ORMDL complex is highly stable and internal rearrangements implicating the C terminal tail might be related to the inhibition of SPTLCs.

The difference between yeast and mammalian ORMDLs are also supported by the fact that in yeast the regulatory mechanism relies on the N-terminal domain of Orms, not present in mammals (see Suppl. Fig. 1 Chapter 1). Thereafter, ORMDLs would sense the different lipid load in the cell and then inhibit SPTLCs due to

internal rearrangements in the complex ORMDL-SPTLC. Based on our results we picture two main mechanisms. Whether these C terminal structural changes that we observed are a reflection of a bigger internal rearrangement in the protein structure is something that cannot be discarded. In addition it could be interesting to study whether this domain could act as a sensor of the lipid environment or even being the inhibitor domain for SPTLCs. Additional functional studies are needed to fully understand the structure-function relationship between SPTLCs and ORMDLs.

1.3. Crosstalk between calcium and sphingolipids

There is an open question regarding the Ca^{2+} imbalance in the ER due to ORMDL3-mediated SERCA inhibition and the possible link to the inhibition of SPTLCs by ORMDLs. In other words, how can an ER resident protein modify two of the main functions of this organelle that apparently are not directly linked? We provide some evidences that build a bridge between these two ER functions. On one hand, our laboratory has observed that ORMDLs co-immunoprecipitate with SPTLCs and SERCA proteins. These proteins are distributed in certain regions of the ER. Specifically, SPTLCs and SERCA are present in MAMs where we have immunolocalized ORMDLs. On the other hand, we have found that the C tail of ORMDLs participates both in SERCA inhibition and in the lipid-dependent ORMDLs' conformational changes. Additional structural and functional studies would be required to demonstrate a crosstalk between the ORMDLs-mediated regulation of *de novo* sphingolipid synthesis and Ca^{2+} homeostasis.

It is possible that altered lipid loading of the cell may alter the biophysical properties of different ER proteins. In this regard, SERCA can be inhibited due to the membrane lipid composition, concretely due to imbalanced phospholipid levels of phosphatidilethanolamide (PE) and phosphatidilcholine (PC)[226–228]. However, PE and PC levels are not affected directly by SPTLC blockade. On the other hand, experiments performed in our laboratory adding myriocin to cells and measuring the ER calcium content showed no differences, evidencing no direct link between SPTLC inhibition and SERCA function. Despite these results are not included in the results chapters of this thesis we found them relevant for the discussion. Finally we find remarkable the fact that both, SERCA and SPTLC1 dysfunction, have been associated to airway diseases[99,229] suggesting a pathological link between the alterations in the two main function where ORMDL3 has been genetically associated to.

1.4. ER stress and unfolded protein response

ORMDL3 affects UPR. Different studies have explored this effect with opposite results depending on the experimental approach. Our group revealed that ORDML3 promotes the PERK pathway[7], others have suggested that overexpression of ORMDL3 enhances ATF6 proteolysis[6,9], and one work claims ORMDL3 does not affect UPR[67]. On the contrary, there is one study that suggests a protective effect of ORMDL3 on UPR induction[3]. Despite of all these diverse effects, one thing is clear, ORMDL3 is directly or indirectly related to UPR. However, to date, no molecular mechanism has explained how ORMDL3 regulates these events. Our opinion is that ORMDL3 is not in the machinery of UPR

signaling pathway. We rather picture that over or dysfunction of this protein leads to ER stress (either by SERCA or SPTLCs inhibition) that in turn activates UPR.

It is known that, under sphingolipid blockade, UPR is activated and induces ceramide synthesis from a catabolic pathway to compensate the lack of ceramide[230]. In this context it would seem likely that ORMDLs, by inhibiting ceramide production could promote ER stress which in turn could induce UPR to restore ceramide levels. There are also evidences pointing to the opposite direction. Yeast studies in a strain DKO for Orm1 and Orm2 present growth defects under Tunicamycin and dithiothreitol, two classical ER stress inducers[1,45,46]. Interestingly, reconstitution of ORMDL3 in these yeast strains rescues the growth defects[45]. These results suggest that the overfunction of SPTLC pathway by the absence of ORMDLs would cause ER stress and trigger UPR.

Regarding Ca^{2+} homeostasis, it is widely described that its imbalance in the ER can also trigger UPR. Ca^{2+} in the ER is tightly controlled as it binds to the luminal chaperones and helps them to allow the proper protein folding. Therefore, inhibition of SERCA pump and the consequent decrease on ER Ca^{2+} content, promotes accumulation of unfolded proteins that activates UPR pathways[54,231,232]. In agreement, SERCA pump is a ER stress-dependent inducible gene[233]. Interestingly, SERCA's decreased activity has been related to induce ER stress in obesity, diabetes type2 and metabolic disease [228,234].

Altogether, we think that a possible link between ORMDL3 and the UPR is the inhibition of SERCA pump and activation of PERK[7].

Another possible pathway is the ORMDL3-dependent induction of SERCA expression by ATF6 nuclear localization[6,9]. With these two evidences we can speculate that ORMDL3 inhibits SERCA pump and this in turn promotes a decreased ER Ca^{2+} load that triggers UPR and in turn enhances SERCA expression as a compensatory mechanism. Nevertheless, we cannot discard STPLC blockade and changes in ER membrane lipid composition as the inductor of UPR. To test this hypothesis, it would be interesting to block SPTLCs in the transgenic mice recently described[9] and to test the ATF6 pathway and SERCA activity (Figure 13).

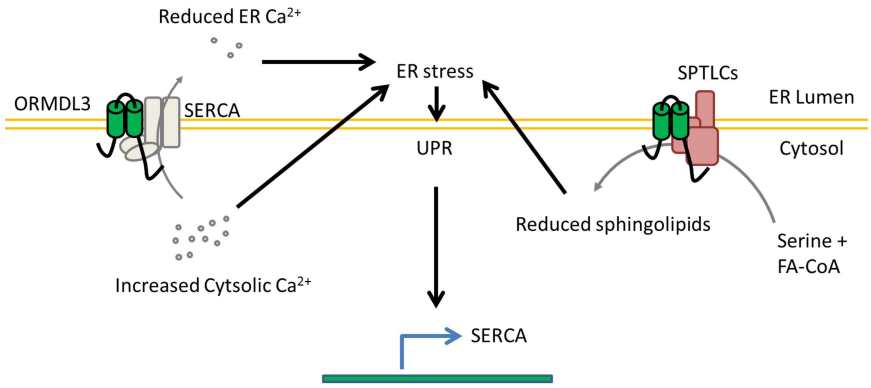

Figure 13: ORMDL3 implications in Ca^{2+} homeostasis, sphingolipid inhibition and UPR activation.

2. ORMDLs in the immune system

ORMDL family is widely expressed [1,2]. Focusing on immune cells, we know that mice challenged with allergic stimuli, ORMDL3 is induced in macrophages and eosinophils but not in neutrophils[6]. Besides, different ORMDL3 expression has been also documented in several cell types, being splenocytes, thymocytes and PBCMs the ones with more basal expression levels compared to epithelial-like cells [3]. Interestingly, it has been also shown that ORMDL3 expression is dependent on STAT6 transcription factor, downstream of a Th2-like polarization status under IL4 and IL13 pathways [6,8,103].

Considering that T cells are main players in the immune response we decided to characterize the regulation of ORMDLs after T cell polarization. Interestingly, ORMDL3 levels remained stable after T cell polarization with the unexpected exception of Th2 subpopulation that presented decreased levels. STAT6 drives Th2 polarization and control IL4 and IL13 expression levels[235,236]. This decreased ORMDL3 levels on Th2 could be explained due to the different models tested, T lymphocytes versus epithelial cells[6]. The total ORMDL amount after T cell polarization is mainly composed by ORMDL3 since ORMDL1 and ORMDL2 are widely downregulated upon T cell polarization. This means that after T cell polarization ORMDL complex would be mainly formed of ORMDL3 subunits, especially in Th1 and Treg cells.

To further understand how these proteins are regulated from a resting state towards an activated one, we tracked ORMDL family upon TCR stimulation. ORMDL2 appeared as the most sensitive isoform and it substantially up-regulated in human and mice samples. This goes in agreement with yeast studies where Orm2 is

induced in a Ca^{2+} dependent manner[51]. Although this would suggest that ORMDL3 is not relevant for T cell activation, we classified our human samples depending on the genotype for the asthma-related SNP rs7216389. Upon this classification, we saw that ORMDL3 is differentially expressed in resting T cells in agreement to what it was described[4]. This basal ORMDL3 increased levels also correlated with a decreased Ca^{2+} entry upon store depletion and with decreased early T cell activation markers. These results confirm our previous results obtained in Jurkat T cells, where ORMDL3 inhibits T cell activation via SOCE decrease (Figure 14).

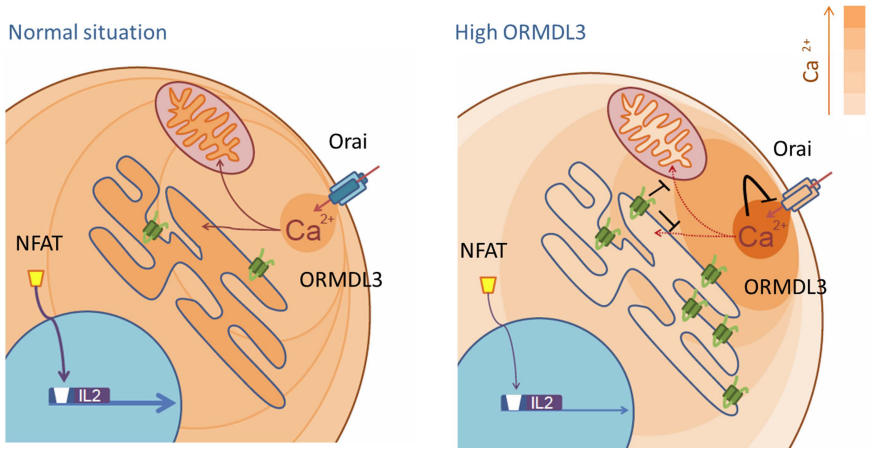

Figure 14: ORMDL3 decreases T cell activation: Under normal situations, TCR engagement promotes Orai protein to open and increase Ca^{2+} in the cytosol. NF-AT will then shuttle into the nucleus to induce IL2 production. High ORMDL3 expression levels increases sCDI by inhibiting store refilling in the ER, via SERCA inhibition and in mitochondria, by inhibiting Ca^{2+} uptake. This reduces NF-AT nuclear translocation and IL2 production.

We have to consider also the possibility that the induction of ORMDL2 at early stages of T cell activation could be related to SPTLCs blockade. Thus, ORMDL2 overexpression correlates with SPTLC1 expression after 24 hours of TCR engagement. This suggests that ORMDL2 induction could control the *de novo* sphingolipid synthesis. Actually, sphingolipid synthesis and T cell physiology are related since blockade of SPTLCs with myriocin has been shown to reduce apoptosis induced cell death (AICD) by preventing sphinganine accumulation in the cell[237–239].

In vivo blockade of SPTLCs with myriocin promotes reduced CD4 population in the spleen and reduced CD4/CD8 cells in the thymus[240]. Interestingly myriocin and its derivative, 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride (FTY720), act as immunosuppressants. Indeed, FTY720 is now used to treat multiple sclerosis[241,242]. However, the immunosuppressive effect after administration of myriocin or FTY720 has been related to endothelial cells. Concretely, in the endothelium, FTY720 acts as an agonist of sphingosine 1 receptor (SP1R) and promotes its downregulation. Sphingosine 1 receptor is responsible for the exit of immune cells from lymph nodes to the blood[243]. In this context, it would be interesting to test in endothelial cells the effect of ORMDLs regarding SP1R expression as they could be controlling immune infiltration. However, this effect might be cell dependent since ORMDL3 overexpression in eosinophils has been shown to promote migration[8].

Finally, similar to the effect of ORMDL3 on T cells, this protein could also have implications on other immune cell types that need CRAC-mediated calcium signals for their activation. Those cell types are mast cells[244–246], natural killer cells and B cells[247]

(reviewed in [248]). Considering the relevance on asthma disease, it would be of great interest to evaluate ORMDL3 impact on mast cell functions. In this respect, one might assume that ORMDL3 expression levels would modify their ability to degranulate [249].

3. ORMDL3 and association studies: Only Asthma? Only ORMDL3?

The association of ORMDL3 and asthma is one of the most independently replicated GWAS. However, the fact that ORMDL3 expression increases asthma risk to around a 1.5 *ods ratio* does not mean it is an asthma cause, we rather think it contributes to an accumulative effect that could sum-up with other traits that could provoke asthma or any other complex diseases.

Two works from the same group have focused on the effect of ORMDL3 overexpression in asthma[6,9]. The most recent one describes a transgenic mouse overexpressing ORMDL3 that develops airway remodeling spontaneously. The authors explain the phenotype based on ATF6 pathway and SERCA induction that would drive matrix remodeling due to the production of matrix metalloproteases from the epithelium. However, another work showed that decreased SERCA activity is related to airway remodeling[229]. Nevertheless, it looks like ORMDL3 is involved in asthma pathology by affecting epithelial cells.

Our studies on T cells claim for a role of this protein in the immune system that might also affect pathophysiological processes. It is true that our data would support more easily a protective role rather an inducer of inflammatory diseases, contrary to what it is demonstrated in the GWAS. Despite of this, the negative effect of ORMDL3 on SOCE/NF-AT axis could also be linked to

autoimmunity. Thus, upon TCR antigen recognition, a series of downstream cascades that imply the recruitment of ZAP-70 that in turn phosphorylates linker for the activation of cells (LAT), causes PLC-γ activation and subsequent calcium mobilization. Knock out mouse models of ZAP-70, LAT, PLC-γ and NF-AT end up in phenotypes that resemble autoimmunity despite the TCR signaling is decreased[115,250,251]. In this direction, we have shown in Jurkat cells and human lymphocytes that ORMDL3 decreases SOCE, a mechanism proposed to modulate Treg development[196]. This, together with the fact that ORMDL3 is the main ORMDL isoform in Treg subpopulation, might unveil a possible effect of ORMDL3 on Treg polarization, creating a connection with proinflammatory pathologies where Tregs are supposed to play a suppressive role.

Furthermore, the effect described on this thesis for ORMDL3 on adaptive immunity can shed light to other diseases linked to its expression levels. Our results exploring the chromosomic region surrounding *ORMDL3* gene agree with the work from Verlaan *et al.*(2009) describing the SNP rs1293623 which exerts a *cis*-regulatory control on the *IKZ3*, *ZPBP2*, *GSDMB* and *ORMDL3* region (17q12-21). One haplotype of this SNP controls in block *GSDMB/ORMDL3*, up-regulating their expression and down-regulating *ZPBP2*. Interestingly, The haplotype associated to increased *GSDMB/ORMDL3* expression is associated to asthma disease, as reported by others, but the one determining a decreased *GSDMB/ORMDL3* levels is associated with Crohn disease (CD), type one diabetes (T1D) and primary biliary cirrhosis (PBC)[211]. This would match nicely with the effect we observe in lymphocyte activation, where decreased ORMDL3 promotes T cell

activation and could explain proinflammatory and autoimmune phenotypes present in T1D, CD and PBC.

In the case of T1D, a destruction of the ß-cells of the pancreas is mediated by autoreactive T cells. Currently, there is an increasing interest in targeting T cell responses that could ameliorate this ß-cell destruction, with special interest on anti CD3 treatments [252–254]. Patients with increased ORMDL3 protein might have less T cell activation reducing the reactivity of these T cells. This could explain why decreased levels of ORMDL3 are related to increased risk to have T1D.

Crohn's disease and ORMDL3 could be linked in different ways. Crohn's disease has been linked to disturbances of the innate immune response (ER stress, Paneth cells, epithelial barrier dysfunction, autophagy and UPR) and to adaptive immune response, like effector CD4 over-activation, Treg development or T cell homing to the gut[255]. Due to the different causes related to the disease, it is difficult to understand the impact of ORMDL3 overexpression or downregulation. On one hand ORMDL3 could have an impact on innate immunity mechanisms by modulating UPR, via SPTLC blockade or Ca^{2+} imbalance, (discussed above) and this could favor an increased UPR under ORMDL3 overexpression. Another possibility that might explain the implication of this protein and Crohn's etiology is the work described in this thesis. Thus, decreased levels of ORMDL3 are genetically linked to this disease and this may alter T cell activation, promoting an overproduction of cytokines and cell migration, hallmarks of the disease[256,257]. A similar explanation would work for PBC, since it is also an autoimmune disease with auto-reactive T cells[258].

However, as discussed before, the fact that GSDMB and ORMDL3 are regulated together may unveil a functional crosstalk between these two proteins with implications in pathology. Expression quantitative loci (eQTL) studies in conjunction with asthma SNPs has revealed that *ORMDL3* has the lowest expression compared to *GSDMB*[102] and *GSDMA*[259,260]. Interestingly, we also observed in our human samples that *GSDMB* is more expressed in TT carriers compared to the asthma protective allele CC. Moreover, as described by Verlaan *et al*, *ZPBP2* is downregulated[211]. These results suggest a collaborative role of the genes placed in this region on the pathological process, where ZPBP2 would be down-regulated and GSDMB/ORMDL3 would be up-regulated to promote asthma disease and to protect for CD, T1D and PBC.

GSDMB is a 411 aminoacid protein present in the cytoplasm of skin and gastrointestinal epithelium[261]. A part of all the linkages to asthma and autoimmune diseases, it has also been related to tumor progression and cancer[262–264].

The other gene affected in this region is *ZPBP2*. This is mainly expressed in gonads and is involved sperm-egg interaction[265] and KO mice studies present subfertility[266].

It would be interesting to co-express in the same cells GSDMB/ORMDL3 proteins and to down-regulate ZPBP2 to see if there is an effect that could potentiate the ones described for ORMDL3 on T cells, or new ones that would shed light on the association studies regarding these two proteins and autoimmune diseases.

It is surprising that, given the high homology between ORMDL family members, only ORMDL3 has been associated to

proinflammatory diseases (Figure 15). This fact would argue against the importance of ORMDL3 as a risk factor and would point at the rest of genes in the region of genetic association[1]. Thus, studies exploring the inhibition of SPTLCs mediated by mammalian ORMDLs, claim that the three members have the same impact on SPTLCs' inhibition, proposing a redundant role of these proteins on the regulation of *de novo* sphingolipid synthesis[52]. Based on this redundant effect, it is surprising that the other two members, *ORMDL1* and *ORMDL2* have not been also linked to any of these diseases. This could be explained in different ways. One explanation would be that there are no strong *cis* regulatory elements controlled by allelic or epigenetic factors in the region of *ORMDL1* and *ORMDL2* enhancing their expression or they have not yet been found. Another possibility is that one specific cell type is particularly sensible to modifications on ORMDL3 expression levels because the other two proteins are not expressed there. This possibility, is rather improbable, since all three members are widely expressed in almost all tissues tested[1,2]. Thereafter, it is also possible that ORMDL3 has special functions blocking SPTLCs and calcium signaling that are not shared with the rest of the ORMDL family.

To sum up, this thesis explores how the function of ORMDL3 in lymphocytes might be related to different pathologies. We propose a mechanism based on calcium signaling that could explain the pathophysiology of different diseases where T cells play a major role. This could underline a more general mechanism behind autoimmune diseases raising the possibility of new molecular approaches to control the immune system dysfunction.

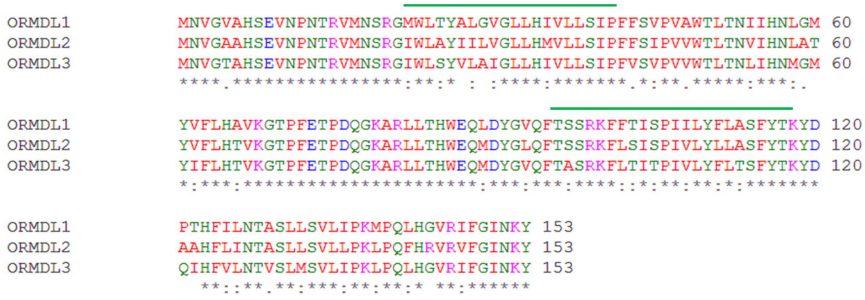

Figure 15: Alignment of ORMDLs reveals high homology: Green lines represent the TM domains. Aminoacid color meaning: Red, Small (small+ hydrophobic (incl.aromatic -Y));
Blue, Acidic; Green, Hydroxyl + sulfhydryl + amine + G; Magneta, Basic – H. (“*”= perfect match; “:” similar properties; “.” more or less similar). Obtained from: https://www.ebi.ac.uk/Tools/msa/clustalw2

vi. CONCLUSIONS

Conclusions

1. ORMDL3 protein negatively regulates T cell activation.

2. Expression levels of ORMDL3 alter mitochondrial Ca^{2+} uptake and modify slow calcium-dependent inactivation of CRAC channel.

3. The first 20 aminoacids of ORMDL3 are necessary and sufficient to produce the inhibitory effect on calcium signaling.

4. ORMDLs expression is induced upon T cell activation, being ORMDL2 the main contributor of this up-regulation at transcriptional level.

5. ORMDLs are differently regulated during T cell polarization.

6. Asthma risk allele of rs7216389 SNP increases ORMDL3 expression levels.

7. SOCE and T cell early activation parameters depend on rs7216389 SNP.

8. Mammalian ORMDLs form homo and hetero-oligomers that in turn interact with SPTLC1 and SPTLC2.

9. The stability of the ORMDL-ORMDL complex and ORMDL-SPTLC binding is independent of the sphingolipid content of the cells.

10. The C terminal tails of ORMDLs rearrange depending on the cellular lipid content.

vi. REFERENCES

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vii. ANNEX

Annex 1

Gene nearby	Crom. Position	SNP id	Change	Associated to	Ref
GSDMA	38128648	rs3859192	C/T (FWD)	Smoking in relation to asthma with AHR	17
				White blood cell count	82
				Asthma	18,19
GSDMA	38121993	rs3894194	C/T (REV)	Asthma and Psoriasis	267
				Smoking in relation to asthma with AHR	17
				White blood cell count	82
GSDMA	38110689	rs17609240	G/T (FWD)	Total number of leukocytes	268
LOC101928947	38089344	rs4794820	A/G (FWD)	Eosinophyl mediated bronchial hyperesponsiveness	269
				Severe asthma	16
				Allergic rhinitis	83
ORMDL3	38082807	rs12603332	C/T (FWD)	Asthma	20,21
				Ankylosing spondilitis susceptibility	270
				Asthma and autoimmune disease	211
ORMDL3	38080912	rs8076131	A/G (FWD)	Childhood asthma and wheeze phenotypes	271
				Childhood asthma	21
				Asthma and autoimmune disease	211
				Asthma and active smoking	17
ORMDL3	38080865	rs4065275	A/G/T (FWD)	Eosinophyl mediated bronchial hyperesponsiveness	269
				Asthma	21,77
				Asthma and autoimmune disease	211
ORMDL3	38077412	rs3169572	C/T (REV)	Asthma	20,21
GSDMB	38075426	rs7224129	A/G (FWD)	Asthma and autoimmune disease	211
				Allergic rhinitis	83

GSDMB	38069949	rs7216389	C/T (FWD)	Childhood asthma	4,72–75
				Asthma	21,272,273
				Childhood asthma, not atopy	76–78
				Asthma and autoimmune disease	211
				Glioma	84
				Allergic rhinitis	83
				Atopic dermatitis	267
				Ankylosing spondilitis susceptibility	270
				Eosinophyl mediated bronchial hyperesponsiveness	269
GSDMB	38066240	rs2290400	A/G (REV)	Childhood asthma	74
				Asthma and autoimmune disease	211
				smoking in relation to asthma with AHR	17
				Type 1 diabetes	10
GSDMB	38066267	rs1008723	G/T (FWD)	Asthma and autoimmune disease	211
GSDMB	38064405	rs11078927	C/T (FWD)	Asthma	95
GSDMB	38062196	rs2305480	C/T (REV)	Childhood asthma and tobacco smoke	274,275
				Childhood asthma	18
				Childhood asthma and wheeze phenotypes	271
				Atopic dermatitis	267
				Primary biliary cirrhosis	276
				Asthma	277
				Ulcerative colitis	3
GSDMB	38057197	rs8069176	A/G (FWD)	Childhood asthma and tobacco smoke	274
				Asthma	277
				Fraction of exhaled nitric oxide values	278
GSDMB/ZPBP2	38051348	rs8067378	A/G (FWD)	Childhood asthma	74
				Asthma	277
				Asthma and autoimmune disease	211

				Ulcerative colitis	3
				cervical cancer	85
GSDMB/ZPBP2	38040763	rs2872507	A/G (FWD)	Childhood asthma	277,279
				Childhood asthma and Inhaled corticosteroids	280
				Crohn disease	11,12
				Crohn disease and colonic transit	15
				Asthma and autoimmune disease	211
				Rheumatoid arthritis	13,14*
				Ulcerative colitis	281
ZPBP2	38028634	rs11557467	G/T (FWD)	Childhood Asthma	74
				Asthma and autoimmune disease	211
				Fraction of exhaled nitric oxide values	278
				Ankylosing spondilitis susceptibility	270
				Primary biliary cirrhosis	276,282
IKZF3	37976469	rs9303277	C/T (FWD)	Childhood asthma	74
				Allergic rhinitis	83
				Childhood asthma and wheeze phenotypes	271
				Ankylosing spondilitis susceptibility	270
				Primary biliary cirrhosis	79,80,81a
				Asthma and autoimmune disease	211

*Suggested as possible

[a]Not only this SNP, but all those tested on 17q12-21