Deregulated inflammasome in Bipolar Disorder (BD): a matter of stress?

Desregulação do inflamassoma na Doença Bipolar (DB): uma questão de stresse?

Dissertação apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra para prestação de provas de Mestrado em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Cláudia Pereira e do Professor Doutor Carlos Bandeira Duarte.

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<div align="right">Para todos os citados, esta dissertação é também vossa!</div>

Table of Content

Table of Content

Abbreviations

.OH - Reactive hydroxyl radical

A

AD - *Alzheimer's disease*

ANK3 - *Ankyrin -3*

AP -1 - *Activator protein 1*

ASC - *Apoptosis -associated speck -like protein containing a C -terminal caspase recruitment domain (CARD)*

ATF4 - *Activating transcription factor 4*

ATF6 - *Activating transcription factor 6*

ATP - *Adenosine -5 -triphosphate*

B

BCA - *Bicinchoninic acid*

BD - *Bipolar disease*

BSA - *Bovine serum albumin*

C

CHOP - *C/EBP homologous protein*

CHUC - *Coimbra University's Hospitals*

CL - *Cardiolipin*

CNS - *Central Nervous System*

CSF - *Cerebrospinal fluid*

D

DAMPs - *Danger -associated molecular patterns*

DB - *Doença bipolar*

DD - *Darier disease*

DISC1 - *Disrupted -In -Schizophrenia 1*

DSM - *Diagnostic and Statistical Manual of Mental Disorders*

E

ECF - *Enhanced chemifluorescence*

EDTA - *Ethylenediamine tetraacetic acid*

eIF2α - *α -subunit of eukaryotic translation -initiation factor 2α*

ER - *Endoplasmic reticulum*

ERAD - *ER -associated degradation*

F

FADD - *FAS -associated death domain protein*

FBS - *Fetal bovine serum*

FBXL2 - *Subunit F -box L2*

FCCP - *Carbonyl cyanide p -trifluorome-thoxyphenylhydrazone*

G

GRP78 - *glucose -regulated protein, 78 kDa*

GRP94 - *glucose -regulated protein, 94 kDa*

GWAS - *Genome -wide association studies*

H

h - *Hours*

H2DCFDA - *2',7' -dichlorodihydrofluoresce-in diacetate acetyl ester*

H_2O_2 - *Hydrogen peroxide*

HBSS - *Hanks' Balanced Salt Solution*

HNE - *4 -hydroxy -2 -nonenal*

I

ICD - *International Classification of Disease*

IMS - *Intermembrane space*

IP3R - *1,4,5 -inositol triphosphate receptor*

iPSCs - *Induced -pluripotent stem cells*

IRE1α - *Inositol -requiring enzyme 1 α*

J

JNK - *c -Jun N -terminal kinase*

K

kDa - *Molecular weight*

L

LPC - *Lysophosphatidylcholine*

LPS - *Lipopolysaccharide*

LTCC - *L -type Ca^{2+} channel*

LTP - *Long -term potentiation*

M

MAMs - *Mitochondria -Associated Membranes*

mCA - *Mitochondrial Ca^{2+} ATPase*

mCU - *Mitochondrial Ca^{2+} uniporter*

Mfn2 - *Mitofusin 2*

mHCX - *Mitochondrial H$^+$/Ca^{2+} exchanger*

min - *Minutes*

mNCX - *Mitochondrial Na$^+$/Ca^{2+} exchanger*

mtDNA - *Mitochondrial DNA*

mtROS - *Mitochondrial ROS*

N

nArgBP2 - *Neural Abelson -related gene -binding protein 2*

NCKX - *Na$^+$/Ca^{2+}/K$^+$ exchanger*

NCX - *Na$^+$/Ca^{2+} exchanger*

NDUFV2 - *NADH dehydrogenase [ubiqui-none] flavoprotein 2*

NF-κB - *Nuclear factor-kappa B*

NLRP3 - *NOD -like receptor family pyrin domain -containing 3*

NLRs - *NOD -like receptors*

O

ODZ4 - *Oz/ten -m homolog 4, TENM4*

P

P2X7Rs - *P2X7 purinergic receptors*

PA - *Phosphatidic acid*

PAMPs - *Pathogen -associated molecular patterns*

PBMCs - *Peripheral blood mononuclear cells*

PBS - *Phosphate buffered saline*

PC - *Phosphatidylcholine*

PE - *Phosphatidylethanolamine*

PERK - *Protein kinase R -like endoplasmic reticulum kinase*

PG - *Phosphatidylglycerol*

PI - *Phosphatidylinositol*

PLs - *Phospholipids*

PMCA - *Plasma membrane Ca^{2+} ATPase*

pro - *IL -18 - Pro -interleukin -18*

pro - *IL -1β - Pro -interleukin -1 beta*

PRR - *Pattern -recognition receptors*

PS - *Phosphatidylserine*

PVDF - *Polyvinylidene difluoride*

R

RE - *Retículo endoplasmático*

ROS - *Reactive oxygen species*

RT - *Room temperature*

RyR - *Ryanodine receptor*

S

SCF - *Skp -Cullin -F box*

SDS -PAGE *- Dodecyl sulphate -polyacryl-amide gels*

SEM - *Standard error of the mean*

SERCA - *Sarcoplasmic/ER Ca^{2+} ATPase*

Sigma -1R *- Sigma -1 receptor*

SM - *Sphingomyelin*

SNPs - *Single nucleotide polymorphisms*

SOD2 - *Superoxide dismutase 2*

T

TBS - *Tris -buffered saline*

TBS -T *- Tris -buffered saline Tween 20*

TG - *Thapsigargin*

TLC - *Thin layer chromatography*

TLRs - *Toll -like receptors*

TM - *Tunicamycin*

TMRE - *Tetramethyl -rhodamine ethyl ester*

TRP - *Transient receptor potential channels*

TRX - *Thioredoxin*

TXNIP - *Thioredoxin -interacting protein*

U

UCPs - *Uncoupling proteins*

UPR - *Unfolded protein response*

UPRE - *Unfolded protein response element*

V

VDAC - *Voltage -dependent anion channel*

WB - *Western Blotting*

WHO - *World Health Organization*

X

XBP1- *X-box binding protein 1*

List of Figures

List of Tables

Resumo

Os problemas de saúde mental constituem a principal fonte de carga económica mundial, estimando-se que o custo global seja superior ao das doenças cardiovasculares, cancro ou diabetes, quando consideradas individualmente. Na União Europeia, as doenças mentais afetam milhões de pessoas e prevê-se que esses números aumentem devido ao envelhecimento da população. A doença bipolar (DB) é um distúrbio mental que se caracteriza por oscilações de humor entre depressão e mania, resultando em danos cognitivos e funcionais que requerem tratamento contínuo. No entanto, os escassos tratamentos atualmente disponíveis apresentam eficácia limitada e o diagnóstico tardio é frequente devido à ausência de marcadores biológicos. Assim, é fundamental compreender a fisiopatologia da DB para a implementação de novos fármacos na prática clínica, bem como para o desenvolvimento de biomarcadores que possibilitem um diagnóstico mais preciso e precoce.

Dado que a DB parece estar intimamente associada ao stresse do retículo endoplasmático (RE) e aos mecanismos para o enfrentar ao longo da sua progressão, propôs-se que as estratégias de resiliência celular desenvolvidas pelas células para lidar com o stresse desempenham um papel crucial na DB. Interessantemente, demonstrou-se que as junções RE-mitocôndria, designadas *"Mitochondria-Associated Membranes" (MAMs)*, são fundamentais nas respostas ao stresse uma vez que modulam a função mitocondrial, a sinalização de cálcio, a via *Unfolded Protein Response (UPR)* induzida pelo stresse do RE e a ativação do inflamassoma, os quais representam marcadores fisiopatológicos da DB. Estas premissas constituíram o ponto de partida para este trabalho, que é focado na "hipótese MAM" proposta pelo nosso grupo para a fisiopatologia da DB, e que pretende investigar o papel do stresse do RE na ativação do inflamassoma NLRP3 e na subsequente libertação de mediadores pró-inflamatórios que certamente sustentam o estado pro-inflamatório associado à DB. As MAMs foram avaliadas como o elo de ligação subjacente ao eixo RE-inflamassoma na DB. Neste sentido, utilizou-se um modelo celular do sistema imune inato, nomeadamente monócitos isolados de doentes bipolares versus controlos saudáveis em condições basais ou expostos a stresse do RE, assim como a linha celular de monócitos humanos THP-1 que foi usada como um modelo in vitro para elucidar as consequências funcionais e morfológicas do stresse do RE.

Os nossos resultados sugerem que o stresse do RE induz a ativação do inflamassoma

NLRP3 em monócitos THP-1 e em monócitos de indivíduos com diagnóstico de DB (fase precoce). Enquanto que nas células THP-1 o stresse do RE atua como sinal 2 para a ativação do inflamassoma, nos monócitos primários de doentes bipolares e de controlos saudáveis atua como sinal 1 e 2. Além disso, apurámos que os monócitos de doentes previamente expostos a LPS são mais sensíveis ao stresse do RE do que os monócitos controlo o que, juntamente com os parâmetros analíticos analisados, sugere que os doentes bipolares são mais suscetíveis a um estado pró-inflamatório. Evidenciámos ainda que os contactos RE-mitocôndria ao nível das MAMs, em particular a chaperona Sigma-1R e a proteína de ligação Mitofusina 2, são afetados pelo stresse do RE tanto nas células THP-1 como nos monócitos de doentes bipolares. Os resultados obtidos nos monócitos THP-1 sugerem que a comunicação RE-mitocôndria em células submetidas ao stresse promove a ativação do NLRP3 através de um mecanismo independente da produção mitocondrial de espécies reativas de oxigénio, o qual parece ser parcialmente mediado pela despolarização da membrana mitocondrial e subsequente externalização de cardiolipina. Também se observou que o stresse do RE provoca alterações morfológicas em monócitos THP-1 compatíveis com RE dilatado, indução da autofagia/mitofagia e modulação da dinâmica mitocondrial.

Este trabalho evidenciou a disfunção das MAMs como um potencial elo fisiopatológico entre o stresse do RE e a ativação do inflamassoma NLRP3 no sistema imune inato, abrindo assim novos horizontes para o desenvolvimento de estratégias terapêuticas ajustadas à DB.

Palavras chave: Doença Bipolar, stresse do RE, Mitochondria-Associated Membranes (MAMs), inflamassoma NLRP3, imunidade inata

Abstract

Mental health problems constitute the largest single source of world economic burden, with an estimated global cost greater than cardiovascular disease, cancer or diabetes individually. In the European Union, mental disorders affect millions of people and these numbers are expected to rise due to population ageing. Bipolar disorder (BD) is a mental illness characterized by mood swings between depression and mania resulting in cognitive and functional impairments that require lifetime treatment. However, few treatments are currently available with limited efficacy and delayed diagnosis is frequent because biomarkers are absent. Therefore, a better understanding of BD pathophysiology is crucial for new drugs design and implementation in clinical settings as well as to develop biomarkers for a more accurate and earlier diagnosis.

Given that BD may be intimately associated with endoplasmic reticulum (ER) stress and coping mechanisms over the illness course, it has been proposed that cellular resilience strategies developed by cells to cope with stress play a key role in BD. Interestingly, it was shown that ER-mitochondria junctions, the so-called "Mitochondria-Associated Membranes" (MAMs), are relevant for stress-related responses by modulating mitochondrial function, Ca^{2+} signaling, ER stress-induced Unfolded Protein Response (UPR) and inflammasome activation, which are pathophysiological hallmarks of BD. These assumptions were the starting point for this work, which is focused on the "MAM hypothesis" proposed by our group for BD pathophysiology that intends to unveil the role of ER stress in NLRP3 inflammasome activation and subsequent release of pro-inflammatory mediators that will certainly sustain a pro-inflammatory status, as well as to evaluate MAMs as the link behind the ER stress-inflammasome axis in BD. For this purpose, a patient-derived cellular model of the innate immune system was used, namely monocytes isolated from BD patients versus healthy controls exposed to basal or ER stressful conditions and, the THP-1 monocytic cell line was used as *in vitro* model to decipher the functional and morphological consequences of ER stress.

Altogether, our results suggest that ER stress induces NLRP3 inflammasome activation in THP-1 monocytes and BD patient-derived monocytes (early-stage). While in THP-1 cells ER stress acts as signal 2 for NLRP3 inflammasome activation, in control and BD monocytes it works as signal 1 and 2. In addition, LPS primed-BD patients' monocytes are more responsive

to ER stress than control monocytes, which together with the analytical parameters analyzed indicate that BD patients are more susceptible to develop a pro-inflammatory status. Furthermore, we uncovered that ER-mitochondria contacts at MAMs, particularly the chaperone Sigma-1R and the tethering protein Mitofusin 2, are affected in THP-1 cells as well as in BD monocytes upon ER stress. Data obtained in THP-1 monocytes suggest that ER-mitochondria communication in ER stressed cells promotes NLRP3 activation by a mitochondrial reactive oxygen species (ROS)-independent mechanism and, might be partially mediated by mitochondria membrane depolarization and subsequent externalization of cardiolipin. It was also observed in THP-1 monocytes that ER stress evoked morphological alterations consistent with dilated ER, autophagy/mitophagy induction and modulation of mitochondrial dynamics.

This work pointed out MAM's dysfunction as a potential pathophysiological link between ER stress and NLRP3 inflammasome activation in the innate immune system thus opening new avenues for the development of fine-tuned therapeutic strategies for BD.

Chapter 1
Introduction

1.1. Mood disorders in Europe

Older people are the segment of the global population with the highest growth, given that the number of individuals over 60 years old doubled since 1980 and the number of older people over 80 years of age is expected to increase more than 4 times (to 395 million) until 2050. According to projections from the European Union, Portugal will be at the top of the list with the highest growth (% of total population) of senior citizens (> 85 years) (EU Ageing Report, 2015). As result of these demographic changes, it is expected that the numbers of older adults with chronic mental illness, as well as the prevalence of associated medical comorbidities will increase.

According to data published in 2010 by the European Brain Council (Gustavsson et al, 2011), mood disorders, which are a group of illnesses where a disturbance in the person's mood is the main underlying feature, affect 33.3 million people in Europe and represent a direct cost per year of 113,405€. However, it is worth noting that the indirect costs associated with decreasing productivity, unemployment and mortality, which are considered the most significant, as well as diagnoses costs, were excluded from the mentioned cost. Recent data from the World Health Organization (WHO) disclosed that the cost of mood disorders and anxiety in the EU is about 170€ billion per year.

Thus, a better understanding of the cellular and molecular basis of mood disorders such as Bipolar disease (BD) is imperative to cope with what is approaching.

1.2. Bipolar Disorder overview

1.2.1. General features

Bipolar Disorder (BD) is a serious mental illness with a chronic relapsing and remitting course affecting 2.4% of the population worldwide that is responsible for loss of more disability-adjusted life-years than all forms of cancer and major neurological conditions (de Sousa, 2014; Merikangas et al, 2011; Murray & Lopez, 1996). According to the WHO, BD is considered the 6th leading cause of disability worldwide. This complex neuropsychiatric disorder is usually diagnosed in adolescence or early adulthood and is characterized by severe and bi-

phasic changes in mood, with patients manifesting intermittent states of mania and depression (de Sousa, 2014; Merikangas et al, 2011; Muneer, 2016). Evidence showing that BD patients spend most of their lifetime depressed led the depressive episodes to be considered the major cause of BD-related disability (Kim et al, 2017; Watkins et al, 2014). This mental disease has been associated with psychiatric comorbidities such as personality disturbance and anxiety disorder, and other medical comorbidities including metabolic and endocrine disorders such as diabetes, obesity and cardiovascular diseases. Besides that, it has been established that BD patients have a higher risk of drug abuse and criminal conducts than the general population (Sayuri et al, 2017). The impact of BD can be devastating, with suicide attempts reported in approximately 23% of patients (Kattimani et al, 2016).

Current knowledge of the BD neurobiology and pathophysiology is still modest (Kim et al, 2017) and with no clear biological markers available, early diagnosis is presently a great challenge to clinicians. Nowadays, the BD diagnosis is established according to clinical interviews.

1.2.2. Diagnosis and treatment options

Nowadays, there are two main criteria for the diagnosis of BD around the world: Diagnostic and Statistical Manual of Mental Disorders (DSM) and the International Classification of Disease (ICD). Only DSM discriminates between BD types I and II. Based on DSM criteria, the BD type I is diagnosed when at least one episode of full-blown mania occurs, accompanied or not by major depressive or hypomanic episodes, while the BD type II is diagnosed when several prolonged episodes and at least one hypomanic episode but no manic episodes occur (American Psychiatric Association, 2013; WHO, 1993). According to the ICD, for a patient to be diagnosed with BD at least two distinct mood episodes must occur, being one a manic or hypomanic episode (Kim et al, 2017).

Actually, the main challenge associated with BD pathology is to overcome the gap between research and clinical practice, which is manifested by polypharmacy reflecting the low effectiveness of the treatments currently available and that can only be overcome by appearance of drugs simultaneously efficient and with low toxicity. Pharmacological therapy is often the first-line BD treatment, followed by psychological (Oud et al, 2016) and psychosocial interven-

tions (Goodwin et al, 2008). For long-term pharmacological treatment, the main goal is to prevent recurrences of mood episodes using mood stabilizers (e.g. lithium, valproate, lamotrigine and carbamazepine) and atypical antipsychotics (e.g. olanzapine, aripiprazole, quetiapine and risperidone) or conventional antidepressants for depressive phases (e.g. selective serotonin reuptake inhibitors or bupropion) (McCormick et al, 2015). Psychosocial treatments include individual psychotherapies, supportive group therapies, education about the disease and focus on treatment adherence and self-care (Geddes & Miklowitz, 2013). Lithium is the most effective long-term therapy for this affective disorder, however, it has many adverse effects and only ~30% of patients respond to maintenance treatment (Malhi et al, 2016; Oruch et al, 2014). Thus, even when submitted to appropriate treatment, majority of BD patients continue to have recurrent mood episodes, residual symptoms, functional impairment, psychosocial disability with high rates of divorce, unemployment, drug abuse and suicide attempting, and significant medical comorbidities such as obesity, diabetes and cardiovascular diseases concomitantly occur. As result of treatment failure, the risk of self-harm and suicide is high in BD patients (Kattimani et al, 2016).

In an attempt to better understand this complex disease, several studies have been developed to elucidate its molecular and cellular mechanisms (Berk et al, 2011). Despite mood disorders are not classified as classic neurodegenerative disorders, increasing evidence suggests that BD pathophysiology is associated with disturbances in brain plasticity, including dysfunction of synapses and circuits (de Sousa, 2014).

1.3. *Impaired cellular resilience underlying BD pathophysiology*

After the proposal of a biological cause for mood disorders, a growing body of evidence suggests that they are associated with regional atrophic brain changes that may be closely related with abnormalities in cellular plasticity, including the resilience of brain cells to resist or adapt to environmental stressors (Walker et al, 2014). Structural neuro-imaging and post mortem studies have highlighted anatomical and neuropathological alterations in BD patients such as ventricular enlargement, decreased levels of neuronal integrity markers and reduction of neuronal density in specific brain areas, suggesting abnormalities in the cellular resilience

towards stressful conditions of neurons and glia cells (Machado-Vieira et al, 2014). These alterations can explain several clinical features such as the progressive shortening of inter-episode interval with each recurrence occurring in consort with reduced probability of treatment response as the illness progresses. Recent genetic studies in postmortem prefrontal cortex samples showing that the EGR3 regulatory unit (regulon), which translates environmental stimuli into long-term changes in the brain, is robustly repressed in BD patients (Pfaffenseller et al, 2016), further support the hypothesis that BD pathophysiology is associated with an impaired response to stress.

Cellular modeling in BD using lymphoblastoid cell lines, fibroblasts, olfactory neuronal epithelium and neurons reprogrammed from induced pluripotent stem cells (iPSCs) has proven useful to understand its biological basis, and potential pathways have been identified, especially in cellular resilience-related mechanisms (Viswanath et al, 2015; Kim et al, 2016a; Stern et al, 2017). The most replicated findings that show consistency with genome-wide association studies (GWAS), brain-imaging and post-mortem brain expression include abnormalities in the endoplasmic reticulum (ER)-related stress responses, mitochondrial function, and Ca^{2+} signaling, which are often reversed *in vitro* by lithium. Furthermore, patient-derived cellular models also support that alterations in glial and immune cell signaling and inflammasome activation, cytoskeleton abnormalities, autophagy impairment as well as induction of oxidative stress and apoptosis, play a relevant role in BD pathophysiology (Bavamian et al, 2015; Bengesser et al, 2016; Chen et al, 2014; Data-Franco et al, 2017; Forstner et al, 2015; Kim et al, 2016b; Kim et al, 2017; Morris et al, 2017; Muneer, 2016; Pfaffenseller et al, 2014; Sun & Shi, 2014; Tobe et al, 2017). The greatest challenge will be to uncover how all these mechanisms are correlated triggering the onset and maintenance of this pathology.

1.4. Ca^{2+} dyshomeostasis and cytoskeleton abnormalities in BD

Over the last few years, several studies have identified common single nucleotide poplymorphisms(SNPs) in CACNA1C and ankyrin-3 (ANK3) in DB patients, implicating these as susceptibility genes for BD development (WTCC, 2007; Ament et al, 2015). These findings support that deregulation of Ca^{2+} homeostasis (**Figure 1**) is involved in BD pathophysiology, as

both CACNA1C and ANK3 encode Ca^{2+} signaling-related proteins: Cav1.2 Ca^{2+} channel are important regulators of Ca^{2+} influx into cells stimulated by ER Ca^{2+} depletion and are critical for normal brain development and plasticity (Harraz & Altier, 2014; Kabir et al, 2017) and ankyrin directly interacts with the ER 1,4,5-inositol triphosphate receptor (IP3R), which is localized at ER-mitochondria contacts, and is responsible for ER Ca^{2+} release (Kline et al, 2008).
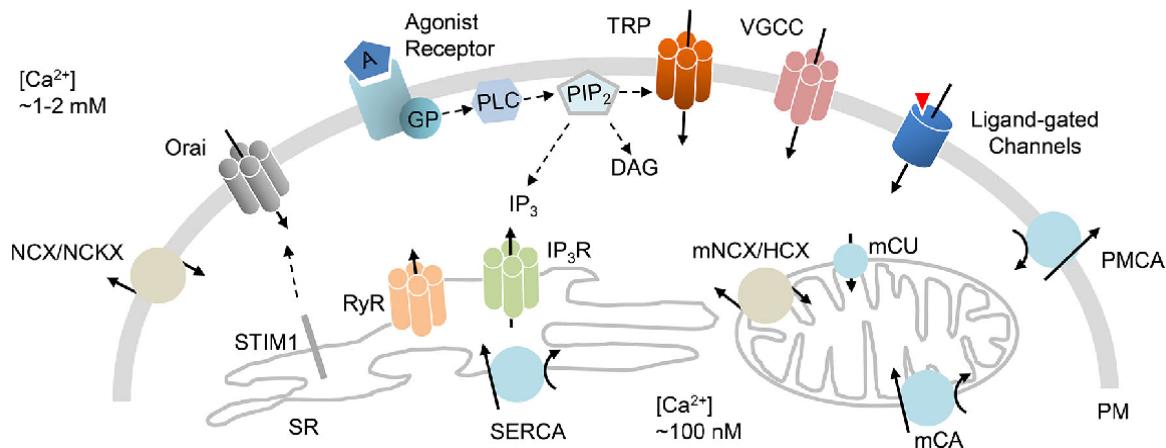
Figure 1. Schematic representation of ion channels and transporters implicated in Ca^{2+} homeostasis. Influx of Ca^{2+} is primarily mediated by VGCC, receptor-mediated Ca^{2+} entry, transient receptor potential channels (TRP), ligand-gated channels, and store-operated Orai channels that are activated by STIM1 protein. Efflux of Ca^{2+} occurs through PM Ca^{2+} ATPase (PMCA), Na^+/Ca^{2+} exchanger (NCX) or Na^+/Ca^{2+}/K^+ exchanger (NCKX). Release of Ca^{2+} from the ER/SR is achieved by IP3R or ryanodine (RyR) receptors. The reuptake of Ca^{2+} into the ER/SR is mediated by sarcoplasmic/ER Ca^{2+} ATPase (SERCA). Mitochondrial Ca^{2+} handling incorporates mitochondrial Ca^{2+} uniporter (mCU), Ca^{2+} ATPase (mCA) or Na^+/Ca^{2+} or H^+/Ca^{2+} exchangers (mNCX, mHCX). From: Harraz & Altier, 2014.

Carriers of the CACNA1C risk polymorphism rs1006737 exhibit greater age-related thickness of cortical brain areas that are widely associated with mood regulation in BD (Soeiro-de-Souza et al, 2017). Furthermore, recent studies demonstrate that the genetic variation rs10761482 of the ANK3 gene affects age-related brain atrophy (Ota et al, 2016). Bioinformatics analysis of differentially expressed proteins, which were previously identified by proteomics in the postsynaptic density from the anterior cingulate cortex of BD patients, further support that BD pathophysiology is associated with changes in the Ca^{2+} signaling (Föcking et al, 2016). Accordingly, L-type Ca^{2+} channel (LTCC) antagonists have been used in BD for over 30 years without becoming an established therapeutic approach. For that, additional genetic, molecular and pharmacological data are required to improve the selectivity, efficacy and tolerability of LTCC antagonists (Cipriani et al, 2016). Moreover, studies using peripheral cells from BD pa-

tients have shown that a Bcl-2 polymorphism contributes to Ca^{2+} dyshomeostasis in this illness through direct interaction with a specific ER IP3R (Machado-Vieira et al, 2011). Therefore, impaired regulation of Ca^{2+} signaling is one of the most reproducible cellular abnormalities in BD and is also supported by findings in patient-derived iPSCs showing alterations of transcripts involved in Ca^{2+} signaling pathways (Chen et al, 2014).

In the last years, several studies have related abnormalities in the cytoskeleton (**Figure 2**) with the pathophysiology of various mood disorders. Neurons derived from individuals with mutations and deficiencies in odd Oz/ten-m homolog 4, TENM4 (ODZ4) genes show abnormal characteristics (Pasca et al, 2011). ODZ4 encodes teneurin, a transmembrane protein that organizes the cytoskeleton (Mosca et al, 2012), which is crucial for several intracellular events such as ER-mitochondria contact and inter-organelle Ca^{2+} transfer (Friedman et al, 2010). Recently, by profiling the proteomics of BD-hiPSC-derived neurons, an aberrant posttranslational modification of collapsin response mediator protein-2, a cytoskeleton-binding protein that is resident on dendritic spines, has been implied in abnormal Ca^{2+} flux (Tobe et al, 2017).

Evidence from clinical studies and animal models support that disturbed microtubule cytoskeleton underlies abnormalities in neuronal cell architecture, especially dendritic complexity and synaptic density changes, which have been inferred in the pathology of several neuropsychiatric diseases such as BD (Marchisella et al, 2016). Aberrant microtubular organization in BD patients might arise from downregulation of microtubule-associated proteins, which in turn may affect Ca^{2+} voltage-activated currents (Solís-Chagoyán et al, 2013). Genome-wide association data from the Psychiatric Genomics Consortium suggests that the deregulation of cytoskeleton remodeling, particularly actin cytoskeleton that impacts on several mechanisms such as endocytosis, phagocytosis, exocytosis, vesicle trafficking, neuronal maturation and migration, neurite outgrowth and synaptic density and plasticity, is implicated in BD pathophysiology (Zhao et al, 2015). Furthermore, a recent study showed that the genetic deletion of neural Abelson-related gene-binding protein 2 (nArgBP2) in mice leads to manic/bipolar-like behavior resembling BD symptoms. nArgBP2 was primarily identified by its interaction with the crucial postsynaptic scaffolding proteins SAPAP3 and Shank3, which have been implicated in the pathogenesis of various mood disorders, such as BD. Lee and Chang revealed that nArgBP2

can modulate spine morphogenesis by controlling actin dynamics and its ablation has been widely associated with inhibition of excitatory synapses (Lee & Chang, 2016). Taken together, these findings suggest that cytoskeletal dysfunction may be involved in BD pathophysiology.
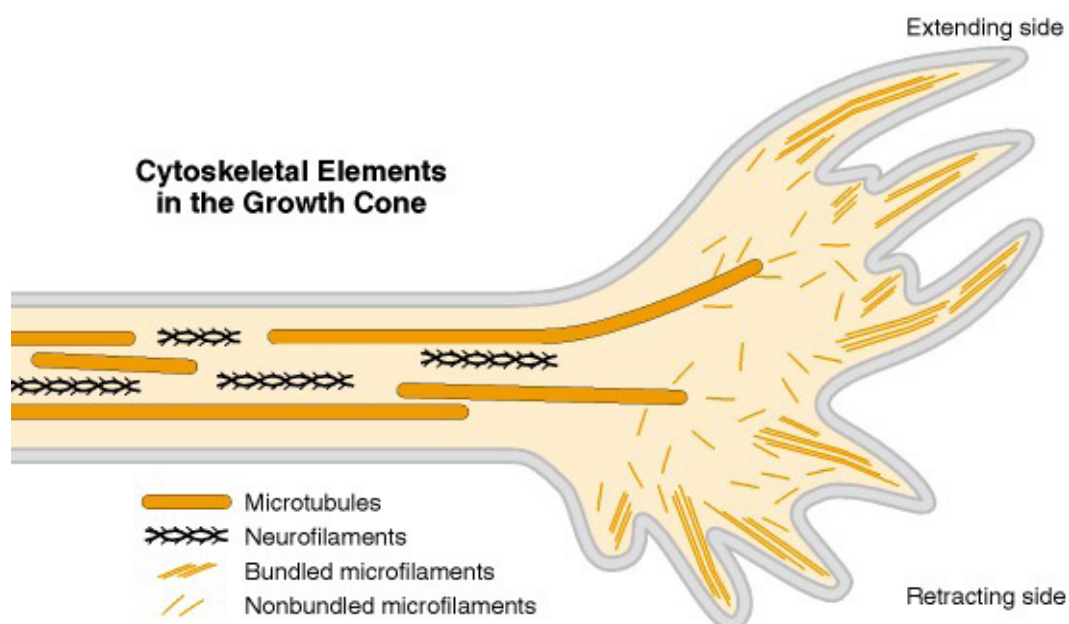
Figure 2. The cytoskeletal elements. The microfilaments are longer and more prominent in the growth cone than in other regions of a neuron. They are bundled in the lamellipodia and in the filopodia. A combination of actin assembly, microfilament cross-linking and myosin motors is required to mediate this movement. In the growth cone, the microfilaments can interact with axonal microtubules, which do not extend to the periphery. These microtubules may be pulled toward the preferred direction of growth and appear to be necessary for net advance. Microtubule movements are thought to be a combination of assembly and contractility. Neurofilaments stabilize the neurite and consolidate advances but they do not appear to be present in the growth cone. From: Kirkpatrick & Brady, 1999.

1.5. Endoplasmic reticulum (ER) in BD pathophysiology

1.5.1. ER: Structural and functional overview

The ER is a eukaryotic organelle positioned throughout the cell by the arrangement of a large and continuous membranous network of interconnected cisternae and tubules (Baumann & Walz, 2001), which outline the ER luminal space from the cytosol. Over the years, ER has been considered as a multifunctional organelle since it is involved in multiple and crucial cellular processes. One of its main functions is clearly to serve as a site for synthesis, folding, and maturation of most secreted and transmembrane proteins. In addition, it has been associated with translocation of proteins across the ER membrane, integration of proteins into the

membrane, folding and modification of proteins in the ER lumen. Beyond its undeniable importance in the life cycle of proteins, ER ensures the synthesis of phospholipids and steroids, and storage of Ca^{2+} ions in the ER lumen (Filadi et al, 2017; Pfaffenseller et al, 2014).

The eukaryotic cells are highly compartmentalized in different organelles with specific functions. However, to maintain cell homeostasis, it is of utmost importance that organelles communicate with each other to modulate cellular processes. The unequalled architecture of the ER, a majestic cisternae-like structure extending from the nuclear envelope to the cell membrane, improves the establishment of inter-organelle communication networks (Spang, 2018). For instance, the mechanism underlying Ca^{2+} balance by ER is one of the examples of its ability to connect with other organelles. As mentioned, the ER allows Ca^{2+} storage, being the greatest reservoir of Ca^{2+} within the cell. On the ER membrane there are Ca^{2+} modulators that when opened allow the efflux of Ca^{2+} ions to the cytosol. As Ca^{2+} is a second messenger implicated in several cellular signaling pathways, the concentration of Ca^{2+} in the cytosol is meticulously regulated (Bootman et al, 2002). When an extensive Ca^{2+} release occurs, various physiological functions that are crucial to maintain cell homeostasis, such as exocytosis, motility, proliferation and gene expression, are affected (Joseph & Hajnoczky, 2007). To avoid a Ca^{2+} imbalance, ER can bind to mitochondria by the interaction between ER-localized IP3R and SERCA and mitochondrial voltage-dependent anion channel (VDAC). In addition to the Ca^{2+} exchange, ER–mitochondria tethering in mammalian cells is also important to ensure lipid transfer from the ER to the mitochondria in order to preserve the mitochondrial functions. ER-Golgi apparatus is another established connection that is important for proteins and lipids exchange. ER can also communicate with endosomes in order to regulate cellular cholesterol levels. Other connections between the ER and organelles such as lysosomes, have been described. Besides that, the ER can also tether with plasma membranes through the ER proteins that have cytosolic domains, which bind lipids and proteins in the plasma membranes (Prinz, 2014).

1.5.2. ER stress and the Unfolded Protein Response (UPR)

The role of ER as a sensor for cellular stress has been supported by several studies in

different cell types (Addinsall et al, 2018; Cao, 2018; Kato & Nishitoh, 2015; Rodrigues et al, 2018). It is also believed that in stress conditions, cells can increase the connection between ER and other organelles in order to trigger a stronger and faster response to cope with stress (Spang, 2018).

Several cellular insults such as oxidative stress, iron imbalance, leakage of Ca^{2+}, protein overload and hypoxia cause the accumulation of unfolded and/or misfolded proteins in the ER lumen triggering ER stress. In response to ER stress, an extremely conserved signaling cascade termed the Unfolded Protein Response (UPR), is activated with the intent to restore ER and cellular homeostasis and proteostasis. Therefore, when activated, UPR triggers a set of transcriptional and translational events to promote cells adaptation to environmental changes in order to reestablish ER homeostasis and preserve cell survival (Decuypere et al, 2011; Deegan et al, 2013).

Canonical mammalian pathways of the UPR signaling (**Figure 3**) involve three specialized ER stress-sensing proteins: protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1α (IRE1α) and the activating transcription factor 6 (ATF6) (Ron & Walter, 2007). Under ER stress conditions, the BiP/GRP78 protein (glucose-regulated protein, 78 kDa), a chaperone that is highly expressed in the ER lumen, dissociates from the ER transmembrane sensors (Bertolotti et al, 2000). The dissociation process induces the oligomerization and phosphorylation of both IRE1α and PERK and the translocation of ATF6 to the Golgi apparatus, where it is cleaved by Site 1 and Site 2 proteases (Ron & Walter, 2007). When activated, IRE1α induces the processing of mRNA encoding X-box binding protein 1 (XBP1), a transcription factor that up-regulates genes that in turn encode several mediators of the ER-associated degradation (ERAD) pathway, organelle biogenesis, and protein quality control (Cox et al, 1993).

Activation of the PERK pathway induces the phosphorylation of the α-subunit of eukaryotic translation-initiation factor 2α (eIF2α), resulting in a decrease of protein load within the ER lumen. When activated by phosphorylation, eIF2α represses the overall protein synthesis and, paradoxically, increases the selective translation of the activating transcription factor 4 (ATF4) mRNA (Harding et al, 1999). ATF4, a member of the transcription factors family bZIP, induces

the expression of several UPR target genes that are involved in antioxidant responses, such as the transcription factor Nrf2 (Harding et al, 2003; Cullinan & Diehl, 2004).

When cells are submitted to ER stress, ATF6 is cleaved at the Golgi apparatus and the re-leased cytosolic domain is translocated to the nucleus, where it regulates the expression of ER chaperones, such as GRP78, ERAD- and autophagy-related genes, and of proteins involved in organelle biogenesis (Haze et al, 1999).
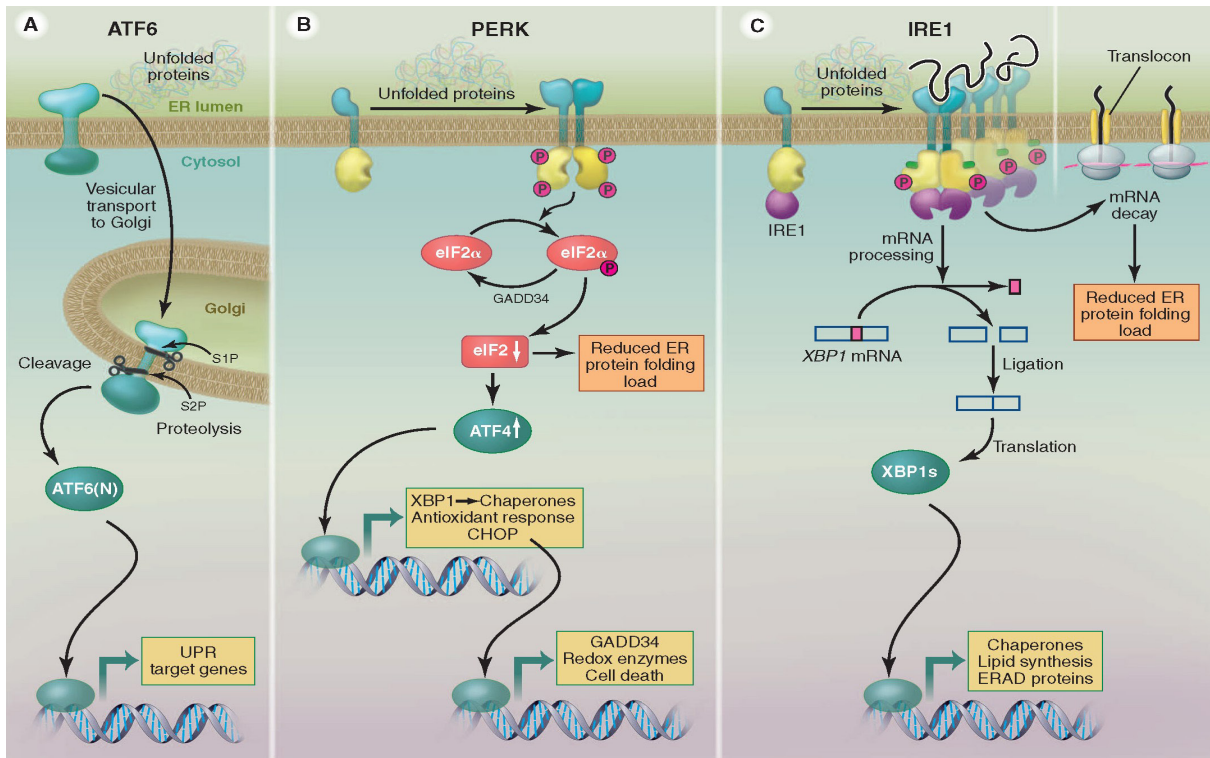
Figure 3. Unfolded Protein Response (UPR) pathway. In the absence of stressful conditions, the BiP chaperone interacts with three ER transmembrane sensors, ATF6, IRE1α, and PERK, maintaining them in an inactive state. Upon ER stress, the BiP chaperone dissociates from the ER transmembrane sensors. The dissociation process induces the dimerization and auto-phosphorylation of PERK, leading to the phosphorylation of eIF2α that induces the selective translation of ATF4 that regulates the transcription of genes involved in antioxidant response and amino acid transporter synthesis. The dissociation process also induces the oligomerization and phosphorylation of IRE1 that results in the cleavage of the XBP1 mRNA to form the transcription factor XBP1s that, in turn, induces the expression of ERAD proteins and chaperones. Besides that, ATF6 is translocated to the Golgi complex, where it is cleaved by Site 1 and Site 2 proteases. Fragments resulting from cleavage of ATF6 are involved in the transactivation of genes encoding ER chaperones and ERAD proteins. From: Walter & Ron, 2011.

Over the past years, a better understanding of ER functions allowed to decipher that UPR is much more than a mechanism to restore ER and cellular homeostasis. Indeed, the ER stress-induced UPR is a well-established mediator of the immune and inflammatory responses

since the UPR signaling pathways play key roles in immune cell differentiation, development, functions, and survival when cells cope with a pro-inflammatory status (Grootjans et al, 2016).

Altogether, the ER can manage cellular stress promoting cell survival through UPR induction that triggers several defense mechanisms to avoid accumulation of misfolded/unfolded proteins. However, the cellular response outcomes are influenced by ER stress levels (Tsang et al, 2010). When ER stress is mild, cells can cope with stress and reestablish normal ER functions. Conversely, when ER stress is excessive and prolonged, this adaptive response fails and a terminal UPR program commits cells to apoptosis (Kim et al, 2008). The major mediators of the UPR that display a key role in ER stress-induced apoptotic cell death are the pro-apoptotic transcriptional factor C/EBP homologous protein (CHOP) and the c-Jun N-terminal kinase (JNK), which when activated induce the expression of numerous pro-apoptotic proteins from the BCL2 family (e.g. Bax) and promote Ca^{2+} release from the ER to the cytosol. As consequence of ER stress-induced Ca^{2+} release, excessive Ca^{2+} uptake into the mitochondrial matrix can occur, and mitochondrial overload in turn induces cytochrome c release and activation of the caspase cascade that leads to DNA fragmentation and cell death (Fribley et al, 2009).

1.5.3. Compromised ER stress response in BD

The ER has been pointed out as the key player in chronic stress, which plays a major role in several brain diseases, including neuropsychiatric disorders (Lindholm et al, 2017). A growing body of evidence has highlighted the crucial role of ER-related stress responses in the pathophysiology of BD. First, different classes of mood stabilizers up-regulate the BiP/GRP78 protein suggesting that ER stress may be involved in BD pathophysiology. Since drugs widely used in BD treatment, such as lithium and valproic acid, can increase ER chaperones expression and activate the unfolded protein response element (UPRE), it can be hypothesized that the mode of action of these mood stabilizers includes UPR activation to reestablish homeostasis (Jadhav et al, 2016). Furthermore, genetic studies demonstrating a significant association between polymorphisms in ER stress-associated genes like GRP78, XBP1 or GRP94 (glucose-regulated protein, 94 kDa) and BD (Bengesser et al, 2016) further support

the involvement of ER in the pathophysiology of this mood disease. As consequence of these studies, the polymorphism in the XBP1 promoter was established as a genetic risk factor for the development of BD, since patient-derived cells containing this mutation have a compromised ability to cope with stress (Kakiuchi et al, 2003). In addition, altered levels of several UPR-related proteins have been described in BD. Under ER stress conditions, an increase of UPR-related proteins, such as GRP78, eIF2α-P and CHOP, was found in healthy controls but not in BD patients and ER stress-induced cell death was significantly higher in patients than in healthy controls. Besides that, monocytes derived from late-stage BD patients showed an altered ER stress response, while monocytes from early-stage patients did not differ from healthy subjects (Pfaffenseller et al, 2014). In accordance, Darier disease (DD) is caused by genetic defects in the gene encoding the sarcoplasmic/ER Ca^{2+}-ATPase isoform 2, an ATPase pump of the ER. Savignac and their colleagues have shown that DD patient-derived cells display biochemical and morphological hallmarks of constitutive ER stress (Savignac et al, 2014). Interestingly, some DD patients develop BD presenting the typical psychiatric manifestations such as depression and manic-like symptoms (Jacobsen et al, 1999). Furthermore, the Disrupted-In-Schizophrenia 1 (DISC1) gene, a genetic candidate for BD development, was shown to modulate the ER-related stress responses through interaction with ATF4, which is abolished by DISC1 substitutions (Malavasi et al, 2012).

Interestingly, most of the neuropsychiatric diseases such as schizophrenia, BD, depression and autism have been related with DISC1 variations as well as an increase of DISC1 insoluble aggregates (Duan et al, 2007). Accordingly, genetic alterations of DISC1 have been directly associated with high levels of protein aggregates (Leliveld et al, 2008; Leliveld et al, 2009) and also with an impairment of mitochondrial axonal transport (Atkin et al, 2012). Mutations in DISC1 and other genes promote protein aggregation by the conversion of misfolded proteins into oligomeric structures (Hetz & Mollereau, 2014; Polajnar & Zerovnik, 2014). Protein aggregates can affect several cellular mechanisms, such as mitochondrial function (Rhein et al, 2009) by slowing axonal transport (Stokin et al, 2005) and promoting oxidative stress (Butterfield & Boyd-Kimball, 2004), and should therefore be removed from the cells (Polajnar & Zerovnik, 2014). However, when the autophagic pathways are impaired, both misfolded proteins

and protein aggregates are not properly cleared from the cell.

Recently, the accumulation of autophagic vacuoles – autophagosomes or autophagolyso-somes – has been reported in several neurodegenerative diseases such as Amyotrophic Lateral Sclerosis suggesting that lysosomal dysfunction might be involved in the pathophysiology of chronic illnesses (Cai et al, 2009; Ikenaka et al, 2013). Accordingly, lithium has been used in the treatment of some neurodegenerative diseases due to its properties as macroautophagy inducer (Calamini & Morimoto, 2012; Polajnar & Zerovnik, 2014). Considering the fact that lithium is widely used in BD treatment as a mood stabilizer to avoid bipolar-like behavior such as depression and apathy (Polajnar & Zerovnik, 2011) and concomitantly triggers macroautophagy, it is possible to hypothesize that this lysosome-dependent protein degradation pathway is deregulated in BD. However, it is worth noting that this is an indirect evidence, indicating that further studies are required. Furthermore, macroautophagy impairment can contribute to the alteration of the ER stress response in BD patients. Indeed, UPR induction under ER stress conditions activates the macroautophagy system to combat the accumulation of misfolded/unfolded proteins and relief cellular stress and avoid apoptotic cell death (Kincaid & Cooper, 2007).

1.6. Mitochondria in BD pathophysiology

1.6.1. Mitochondria: Structural and functional overview

Mitochondria are small organelles playing a central role in cell metabolism under physiological conditions. They are the main energy providers by converting metabolites to adenosine-5-triphosphate (ATP). Based on the chemiosmotic theory, most of the ATP produced by ATP synthase arises from the electrochemical gradient generated by the electron transport chain across the inner membranes of mitochondria (Hroudova & Fisar, 2011). Furthermore, mitochondria are also key organelles in diverse pathophysiological contexts, such as the regulation of free radicals production, Ca^{2+} homeostasis and redox signaling, and also take part in the intrinsic pathway of apoptosis (Hroudova & Fisar, 2011; Scaini et al, 2016). Besides that, mitochondria are involved in the modulation of neuronal activity, neuroplasticity and mor-

phogenesis because their distribution and activity are key factors for the normal neuronal development and synaptic plasticity. For instance, mitochondria regulate the neurotransmitter exocytosis and ion homeostasis in presynaptic nerve terminals (Hroudova & Fisar, 2011; Li et al, 2004).

Over the last years, mitochondria have been pointed out as a central hub of immune cell regulation (**Figure 4**), with mitochondrial outer membrane functioning as a signaling platform to trigger innate immune responses. In addition to their crucial roles in cellular metabolism and programmed cell death, mitochondria contribute to innate immune activation following cellular damage and stress through reactive oxygen species (ROS) production, metabolite availability, and as scaffolding for protein interaction. Indeed, mitochondria have been highlighted as key participants in the organism defense against pathogenic invaders.
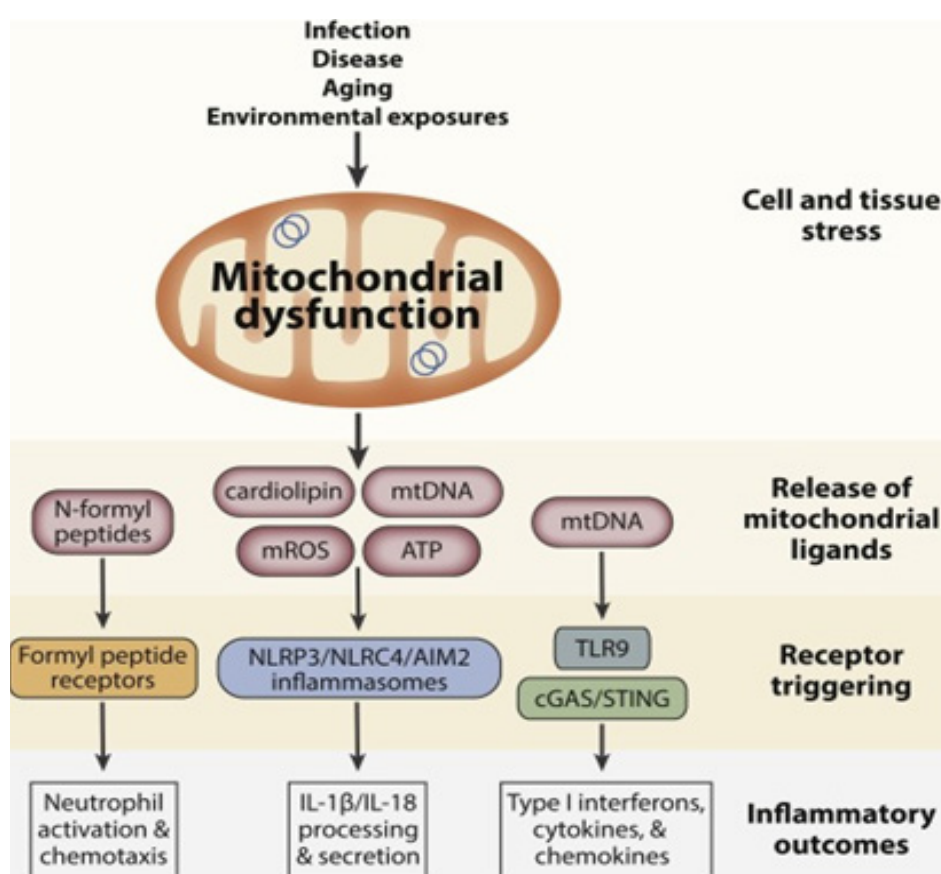
Figure 4. Mitochondria as a key participant in innate immune pathways. In addition to regulate antiviral signaling and antibacterial immunity, mitochondria are also important drivers of inflammation caused by sterile insults. Aging, diseases and environmental exposures can also induce the release of mitochondrial constituents. When mitochondrial integrity is compromised, the mitochondrial ligands released interact with pattern recognition receptors (PRR), triggering inflammation and contributing to the pathology of several diseases. From: West, 2017.

In addition, mitochondria are also important drivers of sterile inflammation caused by injury. Aging, diseases and environmental exposures can also induce the release of mitochondrial constituents, namely mitochondrial DNA (mtDNA), mitochondrial ROS (mtROS), cardiolipins and ATP, which interact with pattern recognition receptors (PRR), triggering immune and inflammatory responses, and contributing to the pathology of several diseases (Weinberg et al, 2015; West, 2017; West, et al, 2011).

1.6.2. *Mitochondrial dysfunction in BD*

Impaired mitochondria function can arise from various factors, including mutations or polymorphisms in mtDNA, deregulation of free radicals production leading to oxidative stress, altered phospholipid metabolism and glycolytic shift, ATP synthesis decrement and changes in Ca^{2+} homeostasis (Hroudova & Fisar, 2011; Scaini et al, 2016). Several findings have implicated mitochondrial dysfunction in the pathophysiology of different diseases, such as cardiovascular diseases, neuromuscular neuropathies, and neurodegenerative and neuropsychiatric disorders (Hroudova & Fisar, 2011). Indeed, mitochondrial dysfunction has been correlated with the pathophysiology of various mood diseases, in particular BD (Cikankova et al, 2016; Clay et al, 2011; Hroudova & Fisar, 2011; Scaini et al, 2016). Kato and Kato were the first to propose the mitochondrial dysfunction hypothesis for BD pathophysiology based on alterations found in BD patients, such as mtDNA abnormalities and amino acid substitutions of specific candidate genes. For instance, the mtDNA polymorphisms 5178C and 10398A present in BD patients are associated, respectively, with decreased brain intracellular pH and altered intracellular Ca^{2+} signaling (Kato & Kato, 2000). In addition, other findings in the brain of BD patients have supported the Kato and Kato hypothesis, including decreased levels and activity of nuclear-encoded subunits of mitochondrial respiratory complexes, decreased pH and altered oxidative phosphorylation (Norkett et al, 2016). Increased cerebral lactate levels in BD patients suggest a shift from aerobic to anaerobic metabolism, further supporting the mitochondrial dysfunction hypothesis in this mood disorder (Brady et al, 2012). Changes in the expression of mitochondria-related genes also corroborate mitochondrial impairment in BD. Maeda and colleagues showed that lymphoblasts from carriers of the DISC1 risk haplotype for BD exhibit

decreased DISC1 levels when compared with lymphoblasts from healthy controls (Maeda et al, 2006). Scola and colleagues suggest that BD patients with differential mitochondria gene expression may be more prone to abnormalities in the electron transfer process (Scola et al, 2013). The expression of NADH dehydrogenase [ubiquinone] flavoprotein 2 (NDUFV2), a nuclear-encoded mitochondrial complex I subunit gene, was found upregulated in a depressed state compared with a euthymic state in BD patients (Munkholm et al, 2015). In addition, dysregulation of the isocitrate metabolism in the mitochondrial citric acid cycle observed in the cerebrospinal fluid (CSF) from BD patients was considered as relevant in BD pathophysiology (Yoshimi et al, 2016).

Interestingly, mitochondrial dysfunction has been identified as the leading cause of cognitive impairment associated with BD. Mitochondria present in the brain are essential for the neurotransmission and neuronal plasticity, which has been defined as crucial for structural and functional adaptations of neuronal circuits to environmental changes and brain damage. One of the major events involved in this dynamic process termed neuroplasticity is the long-term potentiation (LTP) induced, which consists of strengthening of dendritic spines, and new spines/synapses formation. Given that LTP induction has been associated with changes in mitochondrial energy production, an increase of Ca^{2+} pump activity and an enhance of mitochondrial gene expression, mitochondrial distribution and motility determine the occurrence of this dynamic process (Todorova & Blokland, 2017). Since this phenomenon is required for cellular resilience under stress conditions and behavioral adaptation, mitochondrial dysfunction may underlie impaired cellular resilience in BD (Scaini et al, 2016).

Mood stabilizers are used as the first-line in BD treatment because they have modest antidepressant properties and avoid the recurrence of new mood episodes (Davis et al, 2015). However, several studies have contributed to uncover the mechanisms of action of these drugs, revealing that mood stabilizers also interfere with mitochondrial function (Bosetti et al, 2002; Corena-McLeod et al, 2013; Corena-McLeod Mdel et al, 2008; Sun et al, 2006). Taken together, the above evidence support the pathophysiological of mitochondrial dysfunction in BD.

1.6.3. Oxidative stress in BD

Oxidative stress is defined as an imbalance between the production of ROS and antioxidant defenses, and is closely associated with mitochondria since these organelles are the major source of ROS (Halliwell, 2007). Superoxide radicals, which are ROS precursors, are mainly generated by the mitochondrial electron transport chain (**Figure 5**). Although low and intermediate levels of ROS are physiologically relevant to preserve cell survival (Roy et al, 2017), oxidative stress (high levels of ROS) has been pointed out as a possible unifying mechanism for the development of several human pathologies. When the concentration of ROS exceeds the cells responsiveness, the accumulation of oxidative lesions gives rise to ROS-driven stress and reduction of lifespan (Poprac et al, 2017; Schieber & Chandel, 2014).

Oxidative stress has recently been reported to assume a significant role in the pathophysiology and progression of BD. For instance, BD patients show high levels of oxidative stress markers (Scaini et al, 2016; Data-Franco et al, 2017). Lipid peroxidation and nitric oxide levels were shown significantly increased in both red blood cells or serum from BD patients compared to healthy controls (Andreazza et al, 2008a; Brown et al, 2014). An increase of both protein oxidation and nitration was detected in both synaptosomes and mitochondria isolated from BD patients' postmortem prefrontal cortex (Andreazza et al, 2013). The cortex of BD patients demonstrated high levels of the oxidative stress marker 4-hydroxy-2-nonenal (HNE) that is associated with the formation of HNE-protein adducts, which in turn are associated with impaired cellular homeostasis and pathological states (Romano et al, 2017). In addition, several studies have shown increased oxidative damage of nucleic acids in both peripheral blood samples and in postmortem brain samples from BD patients (Andreazza et al, 2008a; Che et al, 2010; Soeiro-de-Souza et al, 2013; Brown et al, 2014). However, it was found heterogeneity between studies in BD patients. For instance, Rosa and collaborators reported significantly lower levels of total glutathione in the plasma from BD patients in comparison with healthy controls (Rosa et al, 2014). On the other hand, Lagopoulos and colleagues (Lagopoulos et al, 2013) showed that young BD patients did not have decreased levels *in vivo* glutathione levels compared with healthy controls. Taken together, the high content of protein carbonyl and lipid hydroperoxide in adult patients (when compared to adolescents) and the increased levels of

an early component of the peroxidation chain in euthymic older patients support the hypothesis of a persistent effect of ROS in BD patients with late age (Andreazza et al, 2015). In addition, it was observed in a recent study that the plasma levels of lipid hydroperoxides were lower in adolescents with fully syndromal BD compared to controls, while levels in the at-risk groups were between healthy controls and fully syndromal BD thus reinforcing a role of oxidative stress in BD risk progression (Scola et al, 2016). Furthermore, Andreazza and colleagues observed that the activity of superoxide dismutase was higher in manic and depressed patients than euthymic patients and controls (Andreazza et al, 2007). Of relevance, the antioxidant properties of numerous mood stabilizers were described (Cui et al 2007; Andreazza et al, 2008b; Bakare et al, 2009; Jornada et al, 2011; Banerjee et al, 2012; de Sousa et al, 2014).
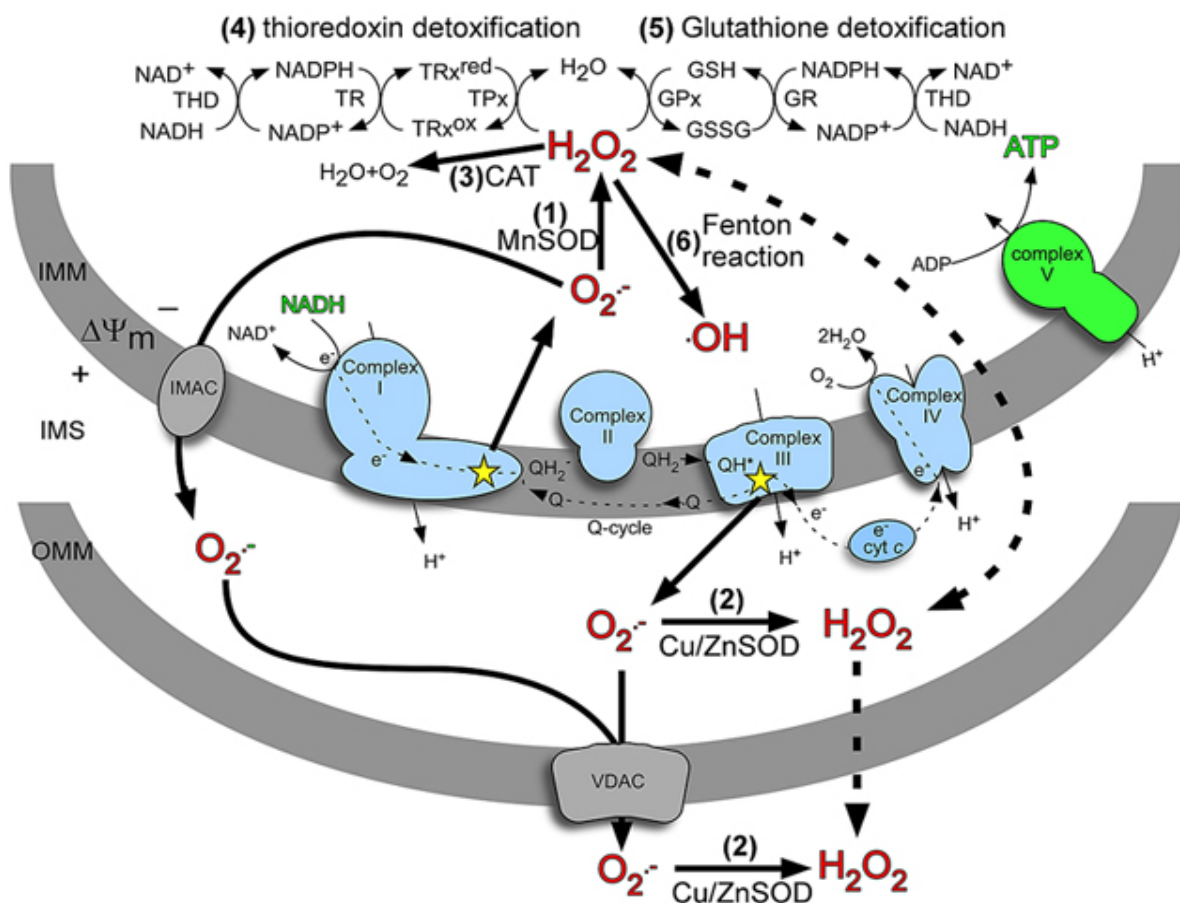
Figure 5. Mitochondrial ROS production and defense. The respiratory chain results in superoxide (O_2.-) generation at complex I and at the intermembrane space (IMS) at complex III (indicated by stars), which is then released to the matrix. O_2.- can be dismutated spontaneously to hydrogen peroxide (H_2O_2) or enzymatically by matrix MnSOD (1) or Cu/ZnSOD (2) in the IMS or cytosol. H_2O_2 can be detoxified in the matrix by three different enzymes: catalase (3), the thioredoxin/thioredoxin peroxidase system (4), or the glutathione/glutathione peroxidase system (5). The fraction of H_2O_2 that does not undergo detoxification reacts with metal ions generating the highly reactive hydroxyl radical (.OH) via the Fenton chemical reaction (6). Whereas H_2O_2 can diffuse freely through membranes (dashed lines), O_2.- can only cross the membranes through ion channels (solid lines). From: Batty et al, 2009.

1.7. Innate Immunity in BD pathophysiology

1.7.1. Innate immune system: General considerations

Numerous disorders, namely brain diseases, have been widely associated with an inflammatory status (Amor et al, 2010). Inflammation is a protective response triggered by the immune system in response to harmful stimuli, such as pathogens, dead cells or irritants. Immune activation within the Central Nervous System (CNS) occurs to remove damaged neurons and infections and is important to assist repair and regeneration of tissues, hence preventing cell death. However, inflammation can also enhance neuronal damage. Thus, the inflammatory response in the CNS may have both neuroprotective and deleterious effects, depending on the circumstances (Amor et al, 2010; Guo et al, 2015).
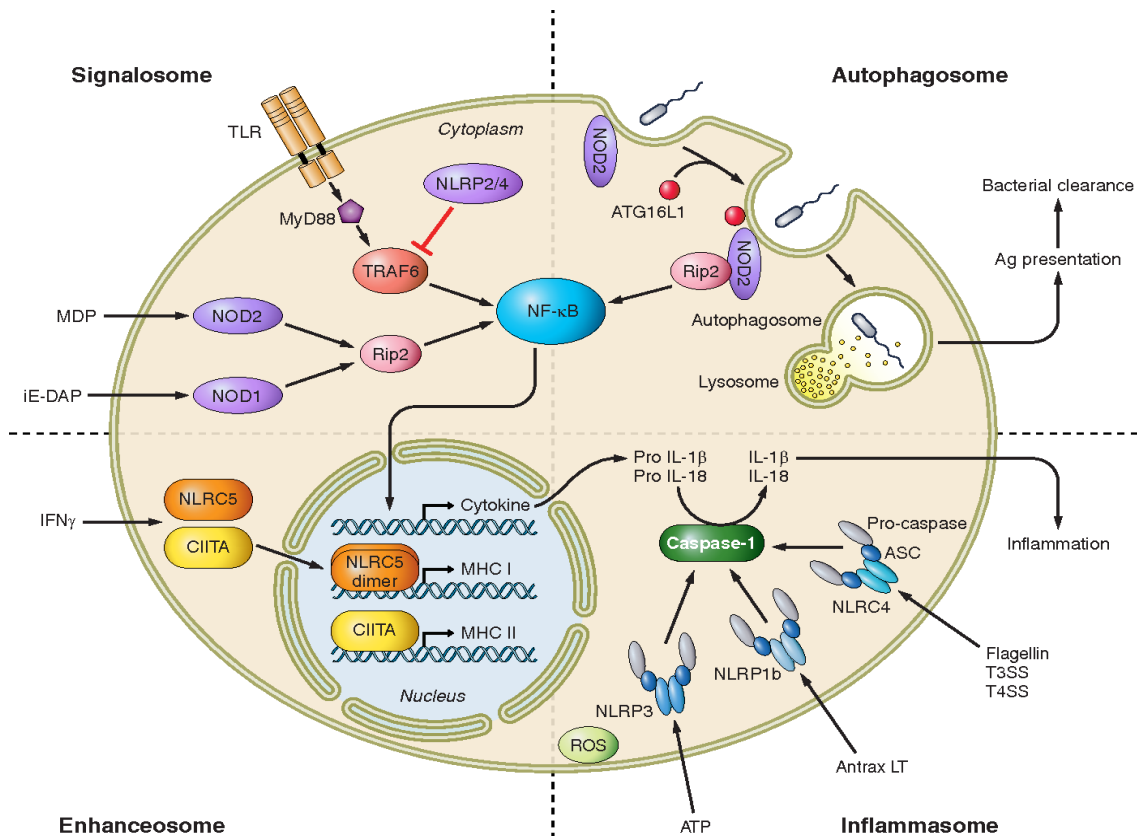
Figure 6. Pattern Recognition Receptors and their activators. The immune cells express pattern-recognition receptors (PRR) to recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Whereas Toll-like receptors (TLRs) recognize PAMPs and DAMPs in the cell surface and in endosomal/lysosomal compartments, the NOD-like receptors (NLRs) are cytosolic sensors. Thus, NLRs acts as second line of defence against pathogenic agents or danger signals that were not detected by TLRs. The recognition of PAMPs and DAMPs by NLRs lead to the inflammasomes assembly.

In response to harmful stimuli, the innate immune system is the first line of defense to trigger an immunologic response. It acts through sensing of pathogen-associated molecular patterns (PAMPs) derived from invading pathogens, and danger-associated molecular patterns (DAMPs) (Walsh et al, 2014). In the CNS, pattern-recognition receptors (PRR) are primarily expressed by microglia, macrophages, and astrocytes. Microglia cells are often termed the "sentinel of brain parenchyma" because they are the primary immune cells of the CNS and respond to pathogens and injury by becoming "activated" (Ginhoux et al, 2013). They can display two different types of PRR (**Figure 6**): Toll-like receptors (TLR), which are membrane spanning receptors, and NOD-like receptors (NLRs) that are cytoplasmic sensors able to oligomerize and form a platform for the inflammasome. In addition to NLRs, the TLRs 3,7,8 and 9 can also trigger inflammasome activation (Hanamsagar et al 2012; Walsh et al, 2014).

1.7.2. *Inflammasome activation*

Inflammasomes are cytosolic multiprotein complexes that are activated to initiate and sustain the innate immune response (Guo et al, 2015; Walsh et al, 2014). They are classically constituted by three main components (**Figure 7**): 1) NLR; 2) the enzyme caspase 1; and 3) an adaptor protein ASC [apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (CARD)], which facilitates the interaction between the two other former components.

According to the variable amino-terminal domain displayed, there are several subfamilies within the NLR family. The members of the NLRP subfamily carry an N-terminal pyrin domain, which interacts with the pyrin domain of ASC to bridge the complex to pro-caspase-1, while the NLR subfamily contains a CARD domain that binds directly to pro-caspase-1 (Walsh et al, 2014). Inflammasome assembly induces the autocatalytic cleavage of caspase-1 and processing of the cytokine precursors: pro-interleukin-1 beta (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into their mature and bioactive forms interleukin-1 beta (IL-1β) and interleukin-18 (IL-18), respectively. These two pro-inflammatory cytokines, which are involved in neuroimmunomodulation, neuroinflammation, and neurodegeneration, can trigger signaling cascades culminating in a type of inflammatory neuronal cell death termed pyroptosis (Guo et al, 2015;

Hanamsagar et al, 2012; He et al, 2016; Walsh et al, 2014).

Focusing on the NLRP3 inflammasome due to the importance garnered in neuroscience, its cytosolic PRR is the NOD-like receptor family pyrin domain-containing 3 (NLRP3). NLRP3 inflammasome is a unique PRR that responds to various activators. There are two well-established pathways for NLRP3 inflammasome activation and an alternative pathway was recently described (Gaidt et al, 2016; He et al, 2016; Walsh et al, 2014).
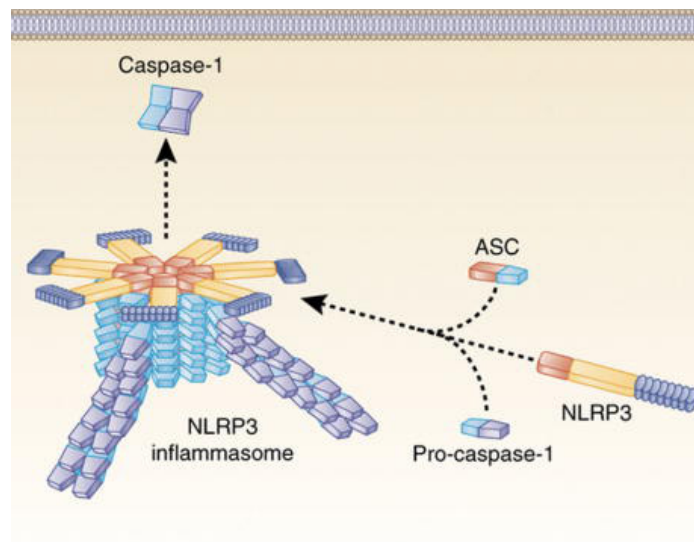
Figure 7. NLRP3 inflammasome components. NLRP3 inflammasome is composed by a cytosolic pattern-recognition receptor (NLRP3), the enzyme caspase-1 and an adaptor protein (ASC), which facilitates the interaction between the former components. Adapted from: Guo et al, 2015.

1.7.2.1. *Canonical inflammasome activation pathway*

The canonical inflammasome activation pathway consists in a two-signal model (**Figure 8**) (Guo et al, 2015; Hanamsagar et al, 2012; He et al, 2016; Walsh et al, 2014). The first signal (priming step) is triggered by TLR ligands or endogenous molecules and involves the activation of nuclear factor-kappa B (NF-κB) or activator protein 1 (AP-1), which upregulate NLRP3 and other inflammasome components (Bauernfeind et al, 2009; Franchi et al, 2009; Malik & Kanneganti, 2017). The second signal induces the assembly of the inflammasomes (activation step) and is provided by diverse stimuli such as ATP, pore-forming toxins, viral RNA, or particulate matter (He et al, 2016). The fact that NLRP3 inflammasome may be activated by different stimuli suggests that it functions as a general sensor of cellular damage and/or stress (He et al, 2016; Walsh et al, 2014). Nowadays, the K^+ efflux is pointed out as the most consensual

trigger for NLRP3 inflammasome activation (He et al, 2016; Walsh et al, 2014). However, many other physiological events, including endosomal rupture, production of ROS and mitochondrial dysfunction, release of mtDNA or cardiolipin, release of cathepsins into the cytosol after lysosomal destabilization, and alterations in Ca^{2+} homeostasis have also been suggested as potential NLRP3 activating stimuli. As NLRP3 agonists do not induce all the above events, the specific mechanism of NLRP3 activation remains still not fully understood (Guo et al, 2015; He et al, 2016; Walsh et al, 2014).

The assembly of NLRP3 inflammasome includes the oligomerization of NLPR3 proteins as well as the sequestration of ASC protein to form a large protein complex, termed "speck", which further allows the recruitment of pro-caspase 1 into the inflammasome. This recruitment is believed to lead to the auto-proteolytic conversion of pro-caspase 1 into its active form (Guo et al, 2015; He et al, 2016; Walsh et al, 2014).
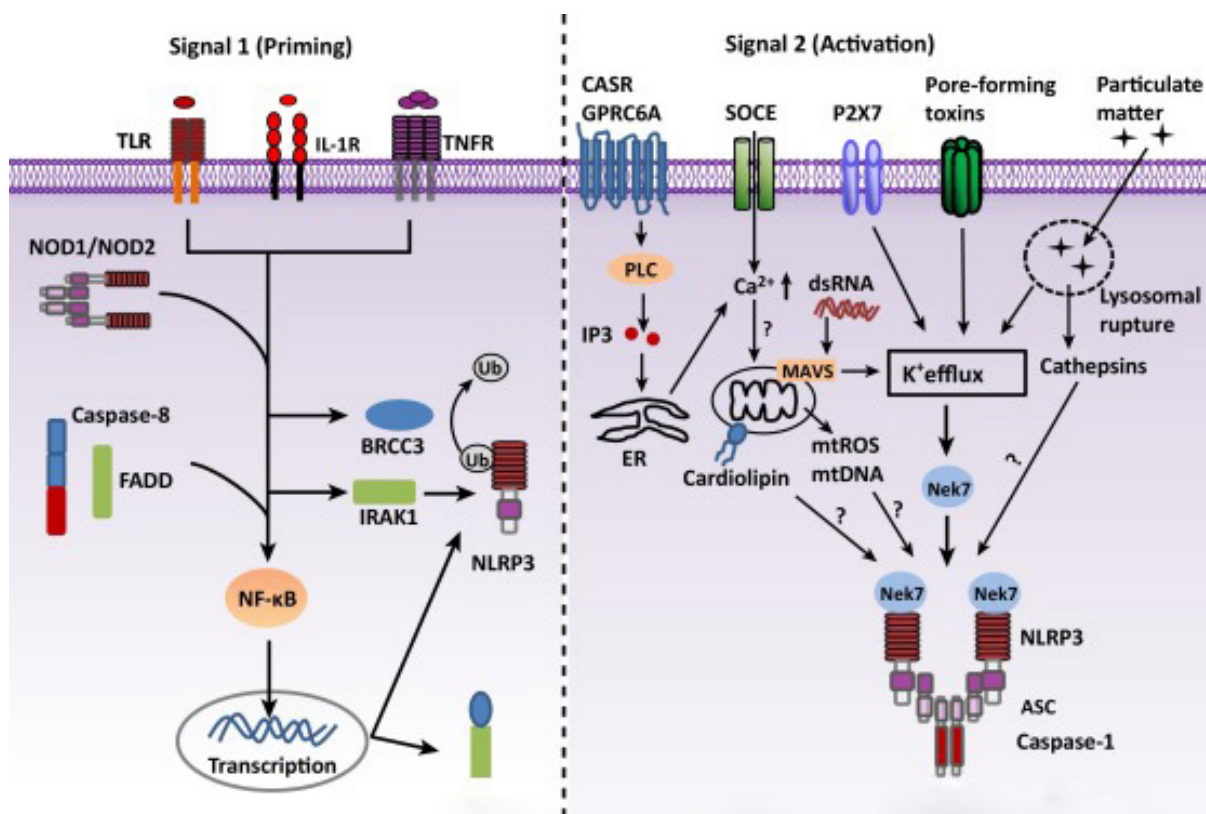
Figure 8. Canonical inflammasome pathway. Signal 1 (priming) is provided by microbial components and leads to the upregulation of NLRP3 and pro-IL-1β through the activation of the transcription factor NF-kB. Caspase-8 and FAS-associated death domain protein (FADD) are involved in the priming via upregulation of the NF-kB activation pathway. Signal 2 (activation) is provided by various stimuli, such as ATP, pore-forming toxins, viral RNA, and particulate matter, leading to the NLRP3 inflammasome activation. Numerous signaling events have been suggested as potential NLRP3 activators. Most NLRP3 activating stimuli induce K^+ efflux, which is necessary and sufficient for NLRP3 activation. From: He et al, 2016.

1.7.2.2. Noncanonical inflammasome activation pathway

The noncanonical inflammasome activation pathway (**Figure 9**) is induced by the lipopoly-saccharide (LPS) released from Gram-negative bacteria into the cytosol by transfection or infection. Once in the cytosol, LPS binds to caspase-11 in mouse (caspase-4 and caspase-5 in humans) triggering its oligomerization and activation (Guo et al, 2015). This connection is not yet fully understood (He et al, 2016). The active form of caspase-11 induces K^+ efflux through the opening of pannexin-1 channel. As mentioned above, K^+ efflux is considered the most consensual signaling event for NLRP3 activation. The assembly of the NLRP3 inflammasome-activates caspase-1 and, subsequently, the secretion of IL-1β and IL-18 (Broz & Dixit, 2016; He et al, 2016).
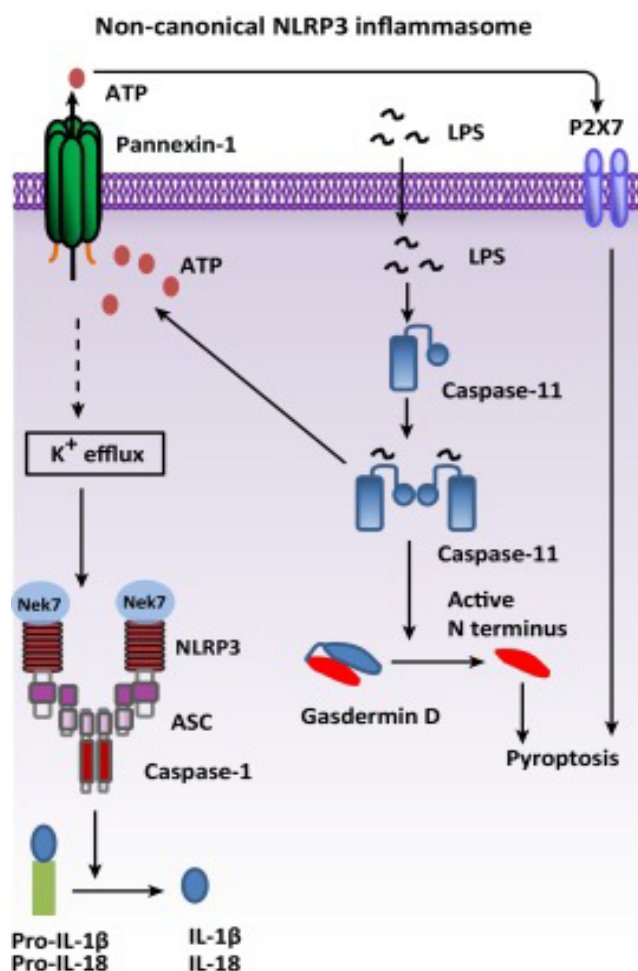
Figure 9. Noncanonical inflammasome pathway. Noncanonical NLRP3 inflammasome activation is only induced by Gram-negative bacteria and is triggered by LPS release into the cytosol through transfection or infection. LPS binds to caspase-1, activating it. Active caspase-11 promotes the opening of the pannexin-1 channel, which induces K^+ efflux required for NLRP3 inflammasome activation and release of IL-1β. The active caspase-11 induces pyro-ptosis through cleavage of the gasdermin D protein leading to disruption of cellular integrity by forming pores on the membrane. The activation of P2X7 purinergic receptors (P2X7Rs) by ATP released from the pannexin-1 channel also contributes to pyroptosis. Adapted from: He et al, 2016.

In parallel, the mature form of caspase-11 can induce the cleavage of the GSDMD-N domain of the gasdermin D protein leading to disruption of cellular integrity by forming pores on the membrane, which in turn induces pyroptosis (He et al, 2015; He et al, 2016; Kayagaki et al, 2015; Shi et al, 2015). Unlike apoptosis, which is characterized by non-inflammatory phagocytic uptake of membrane-bound apoptotic bodies, pyroptosis is a type of cell death that exhibits rapid plasma membrane rupture and release of proinflammatory intracellular contents (Bergsbaken et al, 2009). The P2X7 purinergic receptors (P2X7Rs) are members of the family of ionotropic ATP-gated receptors and when activated by ATP released from the pannexin-1 channel also contribute to pyroptosis induction (He et al, 2016).

1.7.2.3. Alternative inflammasome activation pathway
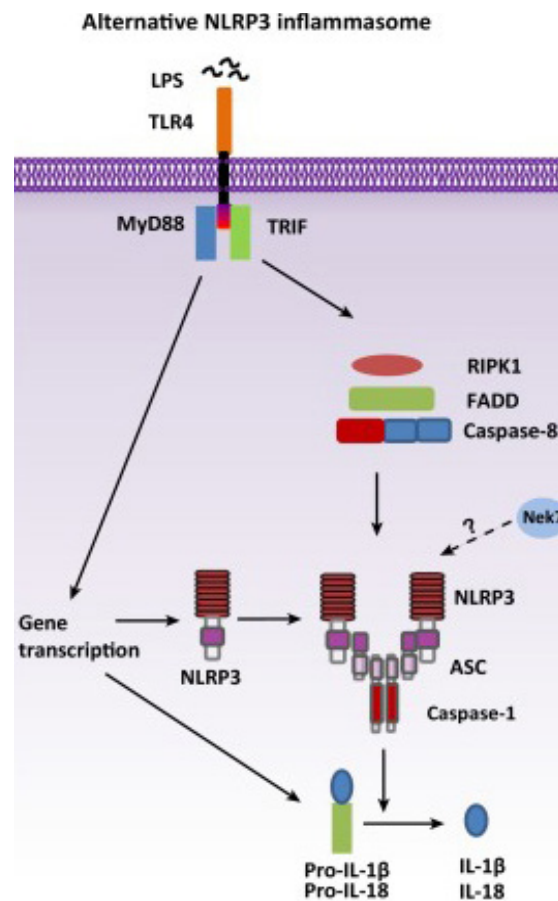
Figure 10. Alternative inflammasome pathway. The alternative NLRP3 inflammasome pathway is activated in human monocytes in response to LPS and it is only induced by TLR4 signaling. In this pathway, K$^+$ efflux is dispensable, the molecules RIPK1, FADD and caspase-8 are required, and the apoptosis-associated speck-like protein containing ASC is not involved. Adapted from: He et al, 2016.

Recently, an alternative pathway for the activation of the NLRP3 inflammasome has been proposed (**Figure 10**). This alternative pathway, which is only activated in human monocytes (Netea et al, 2009; Piccini et al, 2008) in response to LPS, differs from the classical inflammasome activation pathway since it is only induced by TLR4 signaling, without need for a second stimulus (Gaidt et al, 2016; He et al, 2016; Walsh et al, 2014). Additionally, and although the activation of the alternative pathway required both ASC and caspase-1, no evidence of ASC "speck" formation or pyroptosis are observed (Gaidt et al, 2016). Concerning the signaling pathway, the K^+ efflux is not required for NLRP3 activation (in contrasts with canonical and non-canonical pathways) and the molecules RIPK1, FADD, and caspase-8 are upregulated downstream of TLR4-TRIF signaling to activate NLRP3 after LPS stimuli (Gaidt et al, 2016).

1.7.3. BD-associated changes in innate immunity

Over the past decade, the activation of the innate immune system has been implicated in the initiation and/or progression of diseases with a high social and health impact, including metabolic and neurological diseases, such as stroke, neurodegenerative and neuropsychiatric diseases (Libikova et al, 1979; Maes, 1995; Munkholm et al, 2013; Walsh et al, 2014).

Altered levels of cytokines e.g. IL-2, TNF-α, IL-6 and IL-10 are frequently reported both in CNS and peripheral blood of BD patients (Barbosa et al, 2011; Miller et al, 2011; Munkholm et al, 2013; Rao et al, 2010; Soderlund et al, 2011). Given that inflammasomes are the multiprotein complexes that directly mediate the innate immune system's responses to danger signals and pathological circumstances, their activation might be closely associated with the pathological molecular mechanisms underlying BD (Guo et al, 2015; Walsh et al, 2014). Indeed, BD has been consistently associated with immune system alterations. For instance, Soderlund and colleagues reported increased IL-1β levels and diminished IL-6 levels in CSF samples from euthymic BD patients compared to healthy subjects (Soderlund et al, 2011). Since IL-1β secretion is the main readout of inflammasome assembly, the increased levels of IL-1β observed in BD patients strongly suggest that inflammasome activation may underlie BD pathophysiology. There are several findings revealing that chronic diseases, such as diabetes and obesity are highly prevalent in BD patients (Kupfer, 2005; Sayuri et al, 2017). BD-related co-morbidities

mediated by chronic inflammation, strongly support that BD also involves a significant inflammatory microenvironment.

BD patients seem to be particularly vulnerable to the occurrence of mitochondrial dysfunction and, subsequently, to an increase on mitochondrial ROS generation. Elevated ROS levels can positively regulate the levels of pro-inflammatory factors such as IL-1β, caspase-1 and NF-κB (Li et al, 2003). The NLRP3 inflammasome has been pointed out as a redox sensor of ROS production and as the structure behind immune innate-activation. Taking this hypothesis in mind, Kim and colleagues (Kim et al, 2016) proposed to investigate if the NLRP3 sensor is the link between mitochondrial ROS production and immune-activation in BD. Post-mortem analysis of frontal cortex from BD patients found lower levels of complex I and NDUFS7, a subunit of complex I, concomitantly with higher levels of both mitochondrial NLRP3 and ASC, and increased levels of caspase-1, IL-1β, IL-6, TNFα and IL-10 compared with healthy controls (Kim et al, 2016). This study suggests that BD brain immune-activation is associated with mitochondrial dysfunction and NLRP3-inflammasome activation, which is corroborated by findings showing that the induction of inflammatory response in other neuropsychiatric diseases, such as depression and major depression disorder, depends of the NLRP3 inflammasome activation (Jeon et al, 2017; Velasquez & Rappaport, 2016; Walsh et al, 2014). Furthermore, pharmacological inhibition or genetic deficiency of caspase-1 in mice not only diminished depressive- and anxiety-like behaviors but also prevented the depressive-like behaviors triggered in response to chronic stress (Wong et al, 2016). These finding further support the role of inflammasome activation in BD patients that exhibit depressive- and anxiety-like behaviors and demonstrate that it will be interesting to test the protective effect of caspase-1 inhibition in these patients.

Finally, Haneklaus and colleagues strengthened the enrollment of NLRP3 inflammasome in the BD pathophysiology by demonstrating that both NLRP3 inflammasome formation and IL-1β production are regulated by microRNAs that have been implicated in BD (Haneklaus et al, 2012).

Given that inflammasome activation is regulated by specific membranous ER mitochondria microdomains (Raturi & Simmen, 2013), inter-organelle miscommunication can be anticipated

as an upstream event of NLRP3 activation in BD.

1.8. *Mitochondria-Associated Membranes (MAMs) in BD*

1.8.1. *MAMs: Structure and functional overview*

Mitochondria-Associated Membranes (MAMs) are dynamic platforms resulting from the tight association between ER and mitochondria (**Figure 11**) that allow rapid exchange of biological molecules to maintain cellular well-being. The correct ER-mitochondria crosstalk coordinates important functions of the two organelles thus determining key aspects of cell fate. These lipid raft-like ER subdomains, MAMs, which are closely opposed to mitochondria in such a way that the two organelles can physically and biochemically communicate with each other, are crucial for several cellular functions, such as efficient inter-organelle Ca^{2+} transmission controlling mitochondrial bioenergetics and pro-survival/pro-death pathways and determining cell fate under stressful conditions. In addition, MAMs are responsible for regulating mitochondrial dynamics, shape and motility, as well as redox status. Nevertheless, the MAMs function is also extended to lipid metabolism, being phospholipid synthesis one of the major functions of MAMs (Area-Gomez et al, 2012; Missiroli et al, 2018; Rowland & Voeltz, 2012; van et al, 2014).

The last few years have been marked by increased research on the molecular structure and function of MAMs, which provided a greater knowledge of the structural proteins dynamically associated with the ER-mitochondria contacts under physiological and non-physiological conditions. MAMs functions elucidation allowed to understand that their role is much more important than it would be anticipated. As result, MAMs have been envisaged as a central interplay in modulation of several key processes for cell survival, such as ER stress, autophagy, inflammasome signaling and apoptosis (Missiroli et al, 2018; van et al, 2014).

MAMs composition and abundance are highly dynamic being modulated by metabolic demands and cellular insults in order to promote adaptation to different conditions. Under acute or chronic ER stress, the ER-mitochondria crosstalk is affected and may cause metabolic impairment, changes in redox balance and cell death control, which, in turn, may be the source of various CNS disorders, which are associated with morphological and biochemical alterations

in MAMs (Hetz & Mollereau, 2014; Joshi et al, 2016; Filadi et al, 2017). Consequently, alterations of the ER-mitochondria axis have been strongly involved in the onset and progression of several diseases associated with inflammation, including cancer, diabetes, obesity and neurodegenerative disorders (Filadi et al, 2017; Missiroli et al, 2018).
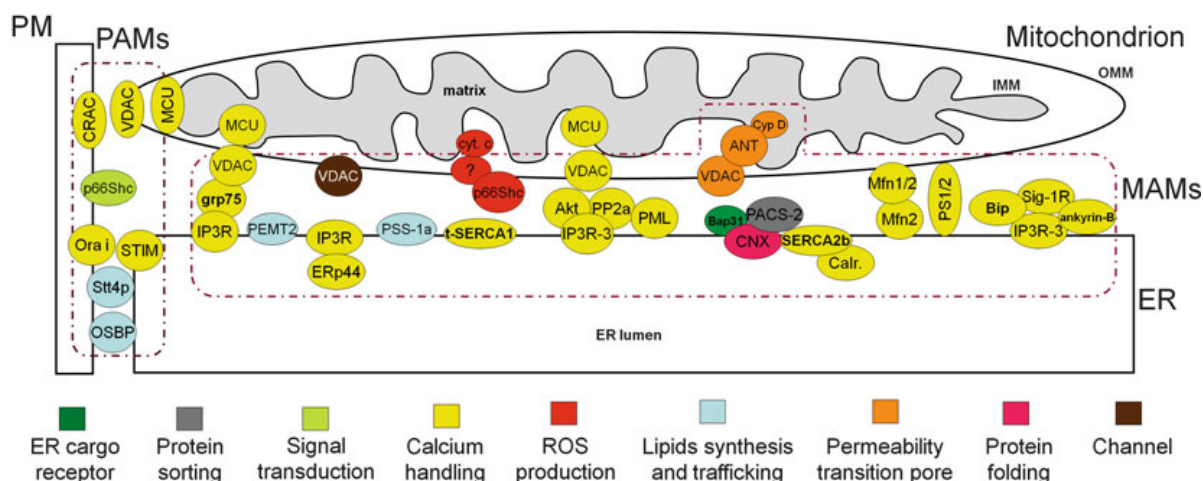
Figure 11. MAMs structure. Schematic view of the inter-organelle interactions and protein composition of the membranes contact sites. Possible contact sites are marked in dotted brown line. ER endoplasmic reticulum, ER lumen endoplasmic reticulum lumen, IMM inner mitochondrial membrane, OMM outer mitochondrial membrane, PAMs plasma membrane associated membranes, PM plasma membrane. From: Bononi et al, 2012.

1.8.2. The link between MAMs and innate immunity

The elucidation of the molecular structure and function of MAMs allowed to unveil that the role of MAMs on innate immunity is much more relevant than it would be initially predicted, with inflammation emerging as one of the pathways controlled by MAMs (Missiroli et al, 2018; van et al, 2014). The first evidence is the subcellular localization of NLRP3 inflammasome-related components under pro-inflammatory conditions. NLRP3 localizes in the ER membrane and cytosol, however, when activated both NLRP3 and ASC are translocated to MAMs to allow inflammasome formation (Zhou et al, 2011). For instance, the mitochondrial antiviral-signaling protein, which is strictly associated with inflammasome activation in antiviral response, recruits NLRP3 to mitochondria. Therefore, it is reasonable that the NLRP3-inflammasome would be formed at the MAM to sense danger signals derived from damaged mitochondria (Subramanian et al, 2013).

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by accumulation

of amyloid-β plaques extracellularly in the brain and associated with ER stress and mitochondrial dysfunction (Costa et al, 2010; Ferreiro et al, 2006). Recent study demonstrated that the NLRP3 inflammasome is activated in AD (Heneka et al, 2013). Moreover, it was shown that both amyloid-β precursor protein and its catabolites are located at MAMs, suggesting that they can modulate MAM-resident components and the ER function (Del Prete et al, 2017; Tramutola et al, 2017).

In the inactive state, another NLRP3-binding partner, thioredoxin-interacting protein (TXNIP), is unavailable to interact with NLRP3 because it is bound to thioredoxin (TRX) antioxidant protein. However, high ROS levels induce conformational changes in TXNIP leading to the dissociation of the TRX–TRXNIP complex, which allows the interaction with NLRP3 and, subsequently, its translocation to MAMs in order to activate NLRP3 inflammasome (Zhou et al, 2010). Specifically, during ER stress, the expression of TXNIP is regulated by two pathways of the UPR signaling cascade, IRE1α and PERK–eIF2α suggesting that TXNIP expression is associated with NLRP3 activation under ER stress. A growing body of findings have suggested that ER stress induces NLPR3 activation by K^+ efflux- and ROS-related mitochondrial events, highlighting the importance of accurate ER-mitochondria communication, for instance to biomolecules exchange (Missiroli et al, 2018; Oslowski et al, 2012).

In addition, under ER stress conditions or in response to oxidative stress, Sigma-1 receptor (Sigma-1R), an intracellular chaperone that resides specifically at the ER-mitochondria interface, prolongs the activation of the UPR signaling pathway IRE1-XBP1, improving cellular survival (Hayashi & Su, 2007; Hayashi, 2015). IRE1 pathway-deficient animals, in which IRE1 cannot be stabilized in MAMs by Sigma-1R, were associated with increased susceptibility to develop bacterial infection when compared to controls (Bischof et al, 2008; Martinon et al, 2010). Furthermore, Mitofusin 2 (Mfn2) protein, which mediates mitochondrial dynamics and is localized on the MAMs to promote mitochondria-ER tethering, has been associated with NLRP3-inflammasome activation. During an infection with viral RNA, Mfn2 provides an excellent scaffold for the NLRP3-inflammasome activation at the MAM, since it directly interacts with NLRP3 and interferon-beta promoter stimulator 1, a mitochondria-associated molecule that recruits NLRP3 to mitochondria in antiviral responses, in a mitochondrial membrane potential

dependent manner (Misawa et al, 2017).

Altogether, the above evidence suggest that ER-mitochondria contact sites at MAMs level play a pivotal role in promoting innate immune inflammation and host defense against invading pathogens. Abnormalities in the ER-mitochondria architecture can be closely correlated with pathological conditions and human diseases (Misawa et al, 2017; Missiroli et al, 2018).

1.8.3. MAMs: Role of Sigma-1R in neuropsychiatric diseases

MAMs-resident chaperone (Sigma-1R) modulates inter-organelle Ca^{2+} signaling and is also important to trigger and to fit anti-stress responses (Hayashi, 2015; Hayashi & Su, 2007).Under stress conditions, this receptor promotes passage of stress signals from the ER to the nucleus through its interaction with various receptors such as the N-methyl-D-aspartate receptors, ion channels, kinases and numerous regulatory key proteins residing on ER, MAM, nucleus or in the cytosol. Based on these complex intracellular actions, the Sigma-1R has been conceptualized as a pluripotent modulator in living systems with pleiotropic protective effects. Recent studies found that the Sigma-1R regulates bioenergetics, free radical generation, oxidative stress, ER UPR and cytokine signaling, as well as morphogenesis of neuronal cells, such as neurite outgrowth, synaptogenesis, and myelination, which can be perturbed by cellular stress (Hayashi, 2015). Sigma-1R activation may therefore control a variety of stress-related cellular systems thus contributing to a cellular defense system that protects the nervous system against chronic stress.

In the last two decades, a considerable amount of clinical data demonstrated the role of Sigma-1R in various pathologies. Ample evidence including the presence of genetic variants within SIGMA1R gene and the interaction of numerous antidepressants with these receptors, suggested a role of Sigma-1R in affective disorders (Hashimoto, 2015; Mandelli et al, 2017). Accordingly, a genetic polymorphism is a risk factor for schizophrenia and Sigma-1Rs levels are significantly reduced in the brain of schizophrenic patients (Ohi et al, 2011). Currently, some drugs (e.g., fluvoxamine, fluoxetine, escitalopram, donepezil, ifenprodil), which have been used in humans, and some endogenous neurosteroids (e.g. dehydroepiandosterone) have high to moderate affinity to Sigma-1R and exert antidepressant-like and neuroprotective

actions supporting their clinical implication in numerous neuropsychiatric diseases (Hashimoto, 2015). The significant advances in Sigma-1R research can be translated into future pharmacological approaches able to control the cellular stress systems based on a better understanding of upstream and downstream intracellular signaling cascades (Ruscher & Wieloch, 2015).

1.8.4. ER-mitochondria miscommunication at MAMs: the "MAM hypothesis for BD"

The above BD-related events are associated with MAMs-related functions and ample evidence show that the MAMs-resident chaperone, Sigma-1R, may be implicated in neuropsychiatric diseases (described in the previous section), therefore, our group proposed the "MAM hypothesis" for BD pathophysiology (**Figure 12**).
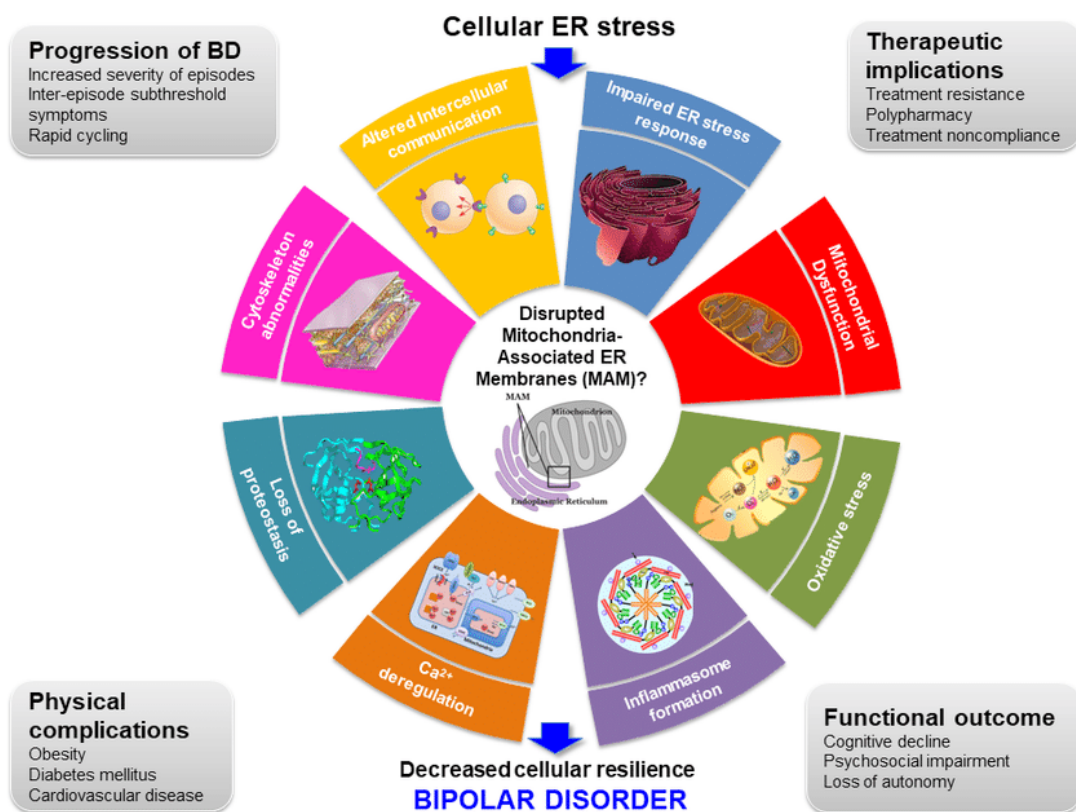
Figure 12. The "Mitochondria-Associated ER Membrane (MAM) Hypothesis" for BD pathophysiology. Patient's cells are chronically exposed to stressful conditions leading to MAMs disruption and subsequent perturbation of cells' ability to cope with stress (cellular resilience), which arises from loss of proteostasis, changes in lipid and calcium homeostasis, dysregulation of intercellular communication, impairment of ER stress responses, mitochondrial dysfunction and oxidative stress, formation of inflammasome and induction of apoptosis, which all have been described in BD. Impairment of these MAM-regulated events decreasing cellular resilience can explain several BD outcomes namely its progressive nature, the functional outcome, the physical complications and also the therapeutic implications, supporting MAMs as promising therapeutic targets for BD. From: Pereira et al, 2017.

This hypothesis considers that ER-mitochondria miscommunication at MAMs is an initial event leading to diminished cellular resilience to stressful conditions and that approaches targeting these specific inter-organelle structures can lead to new treatment strategies and early diagnosis biomarkers (Pereira et al, 2017). MAM hypothesis for BD is supported by studies demonstrating alterations of several MAM-modulated cellular processes in BD patients, namely Ca^{2+} dyshomeostasis and cytoskeleton abnormalities, changes in the ER stress responses, mitochondrial dysfunction and oxidative stress, and inflammasome activation, as described in previous sections.

Chapter 2
Objectives

Bipolar disorder (BD) is a mental illness associated with cognitive and functional impairments that requires lifetime treatment. Unfortunately, few treatments are currently available with limited efficacy and delayed diagnosis is frequent, mainly due to the absence of robust predictive biomarkers. Accordingly, scientific and clinical efforts putting focus on the molecular mechanisms underlying BD pathophysiology have increased, driven by the need to design new drugs and to develop biomarkers for a more accurate and earlier diagnosis.

Several events including impaired cellular resilience, compromised ER stress response, mitochondrial dysfunction and changes in innate immunity have been implied in the initiation and/or progression of BD physiology. Given that all above BD-related events are associated with functions localized at MAMs, our group proposed the "MAM hypothesis" for BD pathophysiology. Based on this hypothesis, this work was designed to unveil the role of ER stress in innate immunity disturbances focusing on NLRP3 inflammasome activation and to investigate alterations of ER-mitochondria contacts at MAMs during ER stress-induced NLRP3 activation, as well as to establish how this pathological axis is modulated in BD. More specifically the main goals of this work are:

(1) to evaluate whether ER stress induction leads to NLRP3 inflammasome activation;

(2) to address the functional and morphological consequences of ER stress on innate immune cells;

(3) to investigate the molecular mechanisms behind ER stress-induced NLRP3 inflammasome activation, with a special focus on NF-κB activation, disturbance of mitochondrial function and compromised ER-mitochondria contacts at MAMs.

To achieve these objectives, the THP-1 monocytic cell line was first used as an *in vitro* model of innate immune cells with the conviction that the molecular mechanisms underlying ER stress-induced sterile inflammation disclosed on these cells would be instrumental to understand the molecular mechanisms underlying deregulation of innate immunity in BD patient-derived monocytes versus age- and gender-matched healthy controls. Tunicamycin and thapsigargin were used as ER stressors in THP-1 monocytes as well as in primary monocytes isolated from early stage BD patients and matched controls.

This Master's research study constitutes the first step in proof-of-concept that will generate

important insights on BD pathophysiology, which ultimately could shed light for novel therapeutic strategies and new diagnostic biomarkers.

Chapter 3
Materials and Methods

3.1. Materials

Cell lines

THP-1 human monocytic cell line (ATCC TIB-202)   InvivoGen (Toulouse, France)

THP-1 cell culture

Fetal bovine serum (FBS)   Gibco, Life Technologies (Paisley, UK)

Penicillin   Sigma-Aldrich Chemical (St. Louis, MO, USA)

RPMI 1640 medium   Sigma-Aldrich Chemical (St. Louis, MO, USA)

Streptomycin   Sigma-Aldrich Chemical (St. Louis, MO, USA)

Primary monocytes isolation and culture

Ficoll-Paque Plus   GE Healthcare (Chalfont St. Giles, UK)

Glutamax   Gibco, Thermo Fisher Scientific (MA, USA)

Heat inactivated FBS   Gibco, Thermo Fisher Scientific (MA, USA)

Human CD14 microbeads   Miltenyi Biotec (Bergisch Gladbach, Germany)

K3EDTA-coated tubes   Vacuette (OPorto, Portugal)

MACS separator (MiniMACS)   Miltenyi Biotec (Bergisch Gladbach, Germany)

MS magnetic column   Miltenyi Biotec (Bergisch Gladbach, Germany)

Non-essential amino acids   Gibco, Thermo Fisher Scientific (MA, USA)

Penicillin   Gibco, Thermo Fisher Scientific (MA, USA)

RPMI 1640 medium, HEPES, no glutamine   Gibco, Thermo Fisher Scientific (MA, USA)

Streptomycin   Gibco, Thermo Fisher Scientific (MA, USA)

Sodium pyruvate   Gibco, Thermo Fisher Scientific (MA, USA)

ER stress inducers

	Sigma-Aldrich Chemical (St. Louis, MO, USA)
Thapsigargin	PanReac AppliChem (Darmstadt, Germany)
	Sigma-Aldrich Chemical (St. Louis, MO, USA)
Tunicamycin	PanReac AppliChem (Darmstadt, Germany)

Fluorescent dyes

Hoechst 33342	Molecular Probes (Eugene, OR, USA)
MitoSOX red mitochondrial superoxide indicator	Molecular Probes (Eugene, OR, USA)
Tetramethyl-rhodamine ethyl ester (TMRE)	Molecular Probes (Eugene, OR, USA)

Kits

Bicinchoninic acid (BCA) protein assay kit	Sigma-Aldrich Chemical (St. Louis, MO, USA)
LEGEND MAX Human IL-1β ELISA kit with precoated plates	Biolegend (San Diego, CA, U.S)

Phospholipid standards

Cardiolipin (CL)	Avanti Polar Lipids (Alabaster, AI, USA)
Lysophosphatidylcholine (LPC)	Avanti Polar Lipids (Alabaster, AI, USA)
Phosphatidic acid (PA)	Avanti Polar Lipids (Alabaster, AI, USA)
Phosphatidylcholine (PC)	Avanti Polar Lipids (Alabaster, AI, USA)
Phosphatidylethanolamine (PE)	Avanti Polar Lipids (Alabaster, AI, USA)
Phosphatidylglycerol (PG)	Avanti Polar Lipids (Alabaster, AI, USA)
Phosphatidylinositol (PI)	Avanti Polar Lipids (Alabaster, AI, USA)
Phosphatidylserine (PS)	Avanti Polar Lipids (Alabaster, AI, USA)
Sphingomyelin (SM)	Avanti Polar Lipids (Alabaster, AI, USA)

Uncouplers of mitochondrial oxidative phosphorylation

Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)	Merck (NJ, USA)

Western blotting

Enhanced chemifluorescence (ECF) reagent	GE Healthcare (Chalfont St.Giles, UK)
Polyvinylidene difluoride (PVDF) membrane	Millipore Corporation (Bedford, MA, USA)
NZY colour protein marker II	NZYTech (Lisbon, Portugal)

Table 1. Source, purification, supplier and reference of the primary and secondary antibodies used in Western blotting analysis.

Antibodies	Source	Purification	Supplier plus reference
Primary Antibodies			
Anti-ATF4	Rabbit	Monoclonal	Cell Signaling Technology (#11815)
Anti-pro Caspase1+p10+p12	Rabbit	Monoclonal	Abcam (#79515)
Anti-CHOP	Mouse	Monoclonal	Cell Signaling Technology (#2895)
Anti-GRP78	Mouse	Monoclonal	BD Biosciences (#610978)
Anti-IkBα	Rabbit	Polyclonal	Cell Signaling Technology (#9242)
Anti-IL-1β	Rabbit	Polyclonal	Santa Cruz Biotechnology (#sc-7884)
Anti-Mitofusin 2	Mouse	Monoclonal	Santa Cruz Biotechnology (#sc-100560)
Anti-NLRP3	Rabbit	Monoclonal	Cell Signaling Technology (#13158)
Anti-Sigma-1R	Goat	Polyclonal	Santa Cruz Biotechnology (#sc-22948)
Anti-XBP1s	Mouse	Monoclonal	Biolegend (#647501)
Anti-p-IKBα	Mouse	Monoclonal	Cell Signaling Technology (#9246)
Anti-β-Tubulin I	Mouse	Monoclonal	Sigma-Aldrich (#T7816)
Secondary Antibodies			
Anti-goat	Rabbit	_	Millipore (#AP106A)
Anti-mouse	Goat	_	GE Healthcare (#NIF1316)
Anti-rabbit	Goat	_	GE Healthcare (#NIF1317)

3.2. Experimental models and treatments

3.2.1. THP-1 cells

The THP-1 human monocytic cell line was cultured and maintained at a cell density between 0.5×10^6 and 1×10^6 cells/mL in RPMI 1640 medium previously supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 25 mM glucose, 10 mM Hepes, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained at 37 ºC in a humidified incubator under an atmosphere containing 5% CO2. Cells were sub-cultured every 2-3 days and kept in culture for a maximum of 2 months.

3.2.2. Primary human monocytes

Human peripheral blood was collected by vein puncture after informed consent from male BD patients (eary stage) and healthy gender- and age-matched controls at Coimbra University's Hospitals (CHUC). Part of the collected volume (2-3 mL) was sent to the CHUC's Clinical Pathology service for analysis of biochemical parameters, namely, inflammatory markers. The study was approved by the ethical committee from the Faculty of Medicine, University of Coimbra.

First, human peripheral blood from BD patients and healthy controls was collected in ethylenediamine tetraacetic acid (EDTA)-treated tubes. Then, peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood by density gradient centrifugation and were then washed to promote removal of platelets.

Briefly, blood collected by vein puncture was diluted with equal parts of sterile phosphate buffered saline (PBS), pH 7.4, and 20 mL were transferred to a 50 mL Falcon tube containing 12 mL of Ficoll-Paque Plus. The tubes were then centrifuged at 1100 g for 20 min, at RT, with the centrifuge brake turned off. Then, the mononuclear fraction, which appear as a dense white band above the layer containing red blood cells and granulocytes, was collected and diluted with 50 mL sterile PBS and centrifuged at 300 g for 10 min, at RT. After discarding the supernatants, pelleted cells were resuspended in 50 mL of sterile PBS and then centrifuged at 200 g for 10 min at RT. After removal of the platelet-containing supernatant, PBMCs were resuspended

in 10 mL of sterile PBS and an aliquot was diluted 10x and counted using an Eve Automatic Cell Counter (NanoEnTeK, USA).

PBMCs were resuspended in the appropriate volume of MACS buffer [PBS, pH 7.2, supplemented with 0.5% (v/v) FBS and 2 mM EDTA], and monocytes were isolated with the human monocyte isolation kit II according to the manufacturer's instructions. For that, the mononuclear fraction was incubated with CD14 microbeads (20 µL of CD14/30 x 10^6 of PBMCs) during 15 min at 4 °C. Pelleted cells were resuspended in 5 mL MACS buffer and then centrifuged at 300 g, for 10 min, at RT. After resuspension in 500 µL MACS buffer, cell suspension was loaded onto a LS magnetic column (previously equilibrated with 500 µL of the same buffer) placed in the magnetic field of a MACS separator and rinsed three times with 500 µL of the same buffer. At this point, the CD14-positively labeled cells were retained in the magnetic field, while the negative cells were eluted. After removal of the column from the magnetic field, the CD14$^+$ fraction was eluted with 2 mL MACS buffer. Finally, the eluted fraction was centrifuged at 300 g for 10 min and the isolated monocytes were cultured at a density of 0.8 x 10^6 cells/mL in glutamine-free RPMI 1640 Medium, supplemented with HEPES, 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2mM glutamax, 1 mM sodium pyruvate, and 0.1 mM non-essentials amino acids. Cells were maintained at 37 ºC in a humidified incubator under an atmosphere containing 5% CO_2.

Given ethical issues limiting the volume of peripheral blood collected from human subjects and multiple collections from the same individual, as well as the low yield of peripheral blood mononuclear cells, the density of plated primary monocytes was lower in comparison with the THP-1 human monocytic cell line, which affected the number of procedures and experimental conditions tested in control and BD monocytes.

3.2.3. Cellular treatments

Lyophilized powder of both ER stress inducers was reconstituted in DMSO to prepare stock solutions: 5 mM thapsigargin and 10 µg/mL tunicamycin.

3.2.3.1. Human monocyte's THP-1 cell line

Once plated, THP-1 cells were immediately treated with tunicamycin or thapsigargin for the time periods and doses indicated in each experimental protocol (**Figure 13**).

For the IL-1β ELISA assay, cells were pre-incubated with lipopolysaccharide (priming) for 24 hours (h) before treatment with tunicamycin (5, 10 μg/mL) or thapsigargin (5, 10 μM) for 8 h or treated with these concentrations of the ER stress inducers for 8 or 32 h (no priming).

Untreated cells or cells incubated with vehicle were used as control.
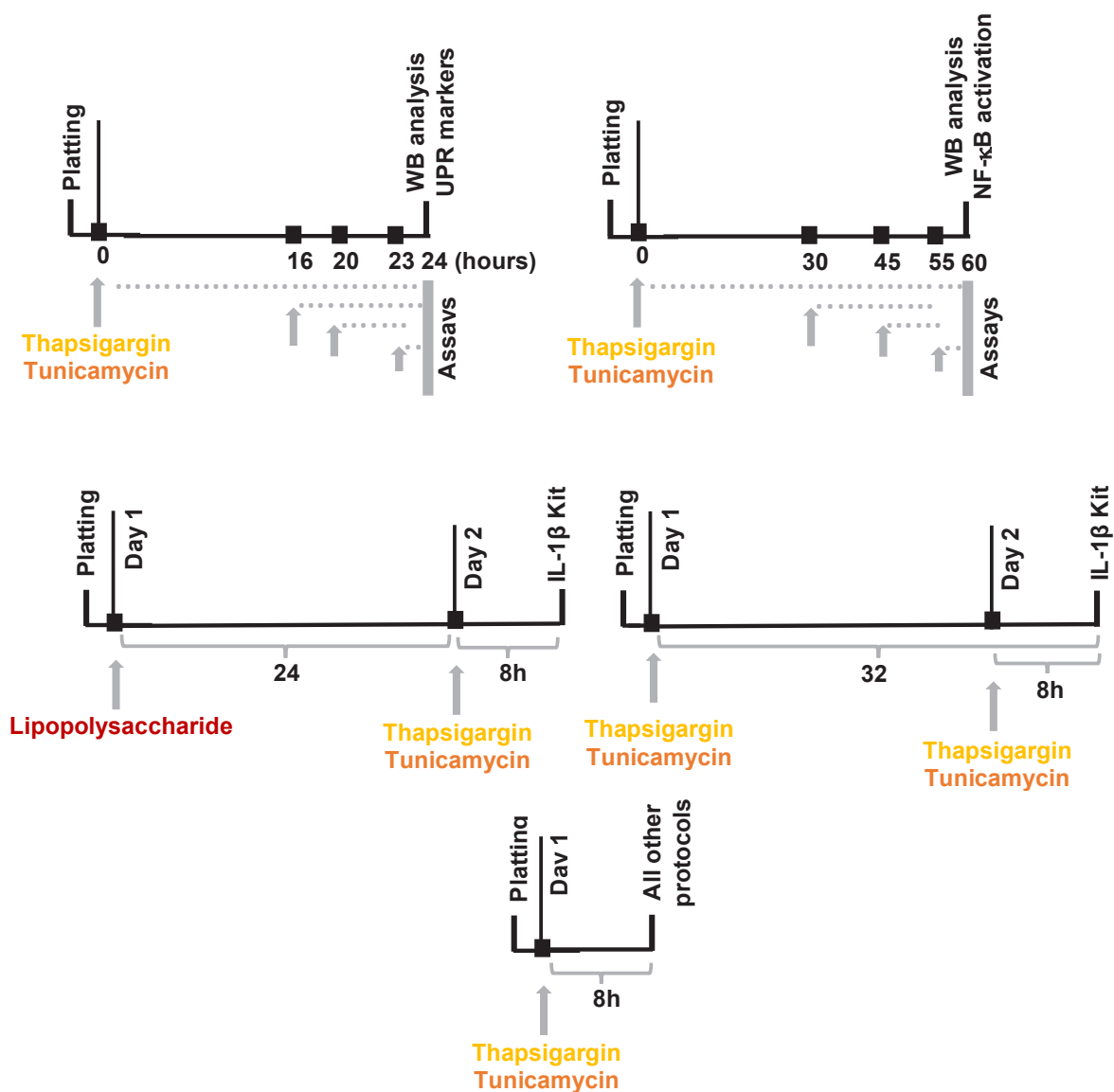
Figure 13. Experimental design showing the procedures performed with THP-1 human monocytic cell line.

3.2.3.2. Primary human monocytes

Once plated, human monocytes were stabilized for approximately 18 h at 37 ºC under an atmosphere containing 5% CO_2. For Western blotting analysis, cells were treated with tunicamycin (5, 10 µg/mL) or thapsigargin (5, 10 µM) for 8 h (**Figure 14**).

In the case of the IL-1β ELISA assay, cells were pre-incubated with lipopolysaccharide (priming) for 24 h prior to treatment with tunicamycin (5, 10 µg/mL) or thapsigargin (5, 10 µM) for 8 h or only treated with similar doses of these ER stressors for 8 or 32 h (no priming).

Untreated cells or cells incubated with vehicle were used as control.
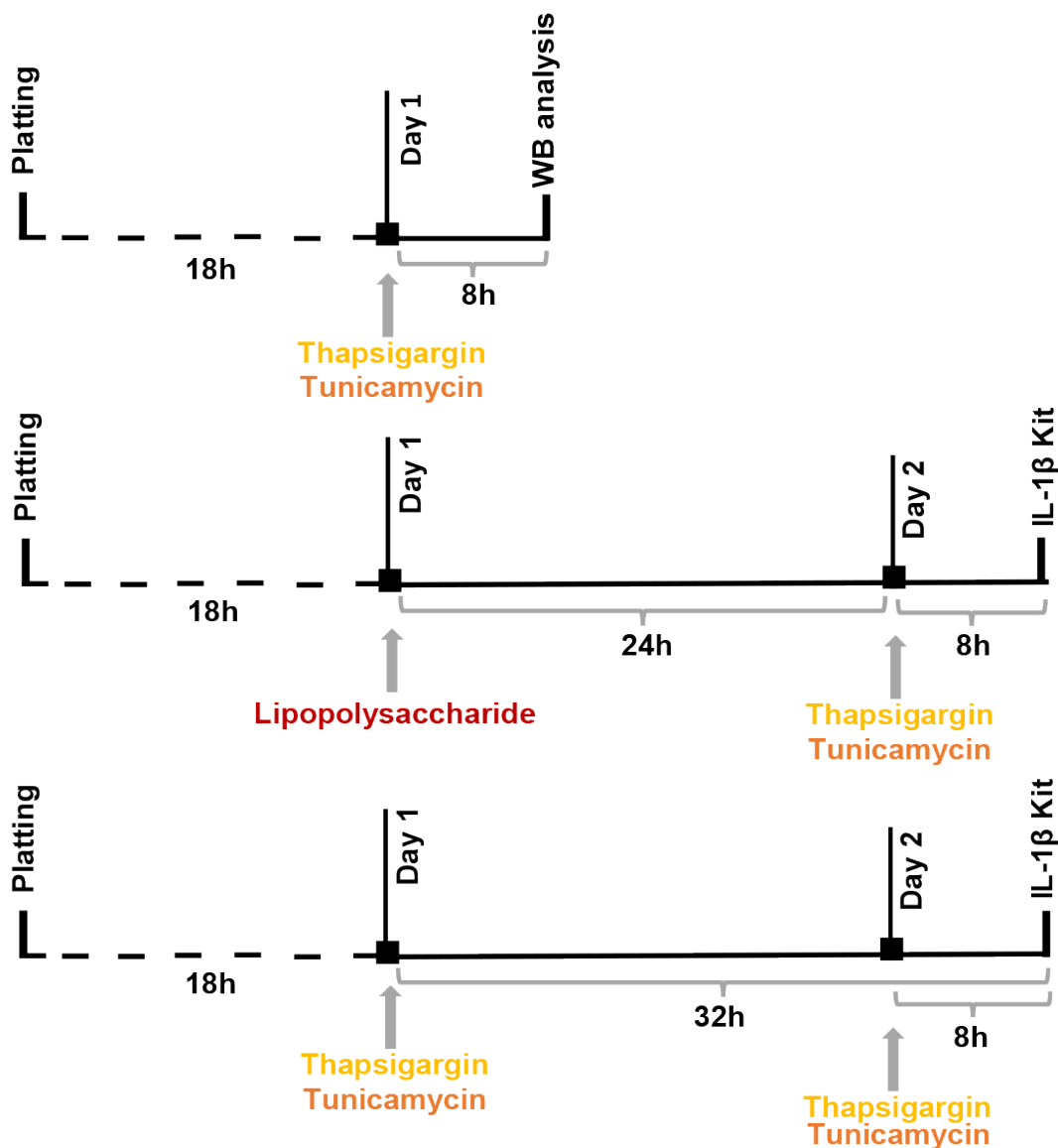
Figure 14. Experimental design showing the procedures performed with human monocytes isolated from human peripheral blood from BD patients and healthy controls.

Table 2. Summary of cell treatments.

Treatments	Function	Abbreviation/Dose
Thapsigargin	ER stress inducer (SERCA Ca^{2+} ATPase inhibitor)	TG (5, 10 μM)
Tunicamycin	ER stress inducer (N-acetylglucosamine transferase inhibitor)	TM (5, 10 μg/mL)
Lipopolysaccharide	Activator of the NLRP3 inflammasome (priming signal)	LPS (1 μg/mL)

3.3. Methods

3.3.1. Cell viability assay

Susceptibility towards ER stress was evaluated in human monocytes' THP1 cells by the resazurin assay, which monitors cell viability based on metabolic activity. Viable cells metabolically active reduce resazurin, a non-fluorescent blue dye, into a highly fluorescent pink dye called resorufin (Ahmed et al, 1994; Prabst et al, 2017).

THP-1 cells were cultured in a 96-well plate at a density of 0.2 x 10^6 cells/well in a final volume of 200 µL. Then, cells were treated with tunicamycin (0.025, 0.1, 1, 5 or 10 µg/mL) or thapsigargin (0.5, 1; 2.5, 5 or 10 µM) for 24 h. Four hours before the end of treatment, resazurin solution was added to each well into a final concentration of 50 µM and incubated at 37 ºC. Finally, the values of absorbance were measured at 570 and 600 nm in a standard spectrophotometer Synergy HT Multi Detection Microplate Reader (BioTek Instruments, USA). Each condition was performed in triplicate, at least, in three independent experiments.

3.3.2. Total cell lysates preparation

To obtain total cell lysates for Western Blotting (WB) analysis, THP-1 cells were plated in 6-well plate at a density of 2.4 x 10^6 cells/well in a final volume of 3 mL. Once plated, cells were treated with tunicamycin (5, 10 µg/mL) or thapsigargin (5, 10 µM) for the indicated time periods. After the incubation time, cells were centrifuged at 300 g for 5 minutes (min) at 4 ºC

to remove the medium and then washed with cold PBS, pH 7.4. Afterwards, cells were lysed in RIPA buffer [50 mM Tris–HCl (pH 8.0), 1% (v/v) Nonidet P-40, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 2 mM EDTA, and 1 mM DTT] freshly supplemented with a protease and phosphatase inhibitor cocktail, and then incubated on ice for 30 min. Nuclei and insoluble cell debris were removed by centrifugation at 4 ºC, at 12,000 g for 10 min. The obtained extracts, which correspond to supernatant fraction, were collected and stored at -80 ºC until further use.

For WB analysis, total cell lysates were also obtained from primary monocytes plated in 12-well plates at a density of 1.2 x 10^6 cells/well in a final volume of 1.5 mL, upon treatment with tunicamycin (5, 10 μg/mL) or thapsigargin (5, 10 μM) for 8 h.

3.3.3. Protein quantification

The bicinchoninic acid (BCA) protein assay was used to quantify the total protein content in cell lysates (description in section 3.3.2). Briefly, this method is based on the conversion of Cu^{2+} to Cu^+ when the first reacts with proteins (Biuret reaction). The concentration of Cu^+ is determined by reaction with BCA and directly correlates with protein amount (Smith et al, 1985).

The BCA Protein Assay Kit was used according to the manufacturer's instructions using a microplate procedure with 25 μL of sample per 200 μL of BCA working reagent. The microplate was then incubated at 37 ºC for 30 min. At the end, absorbance values were read at 570 nm in a standard spectrophotometer Synergy HT Multi Detection Microplate Reader (BioTek Instruments, USA). The protein content of samples was determined by extrapolation using a standard linear regression, which is traced with known concentrations of standard bovine serum albumin (BSA) (Walker, 1994).

Protein concentration in each cell lysate obtained from primary monocytes was also determined by the BCA protein assay, as described above. Samples whose protein amount was not enough to be quantified by the BCA assay were submitted to acetone precipitation to concentrate proteins from the RIPA buffer and the procedure was carried out as described by Botelho and colleagues (Botelho et al, 2010) with modifications. Briefly, 4 x volume of cold acetone was added to each sample. The mixtures were homogenized by vortex and were then incubated

overnight at -20°C. After centrifugation at 15000 g for 10 min, supernatants were carefully discarded, and the pellets were air dried during approximately 20-25 min. Finally, precipitated proteins were dissolved in 1x sample buffer preheated.

3.3.4. Western blotting

After BCA quantification, the concentration of protein in each sample was adjusted to 30 or 40 µg to ensure an equal amount of protein loading. Then, the samples were denatured by adding 2x concentrated sample buffer [5% (w/v) SDS, 0.125 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and bromophenol blue] and heated for 5 min at 95 ºC. Proteins were then separated in 10% (v/v) sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) at 130 V for 60-75 min. After separation, proteins were transferred from the gel to a previously methanol-activated polyvinylidene difluoride (PVDF) membrane by electroblotting using a Trans-Blot Cell wet transfer system (Bio-Rad, USA), at 400 mA, during 3 h and at 4 ºC. After blocking with 5% (w/v) nonfat dry milk in Tris-buffered saline [(TBS): 150 mM NaCl, 25 mM Tris-HCl pH 7.6] containing 0.1% (v/v) Tween 20 (TBS-T) for 1 h at room temperature (RT), membranes were further incubated with the primary antibodies (**Table 3**), prepared in TBS-T with 5% (w/v) milk, overnight, at 4 ºC. Afterwards, the membranes were washed 3 times with TBS-T and incubated for 1 h, at RT, with the alkaline phosphatase-conjugated secondary antibodies in a 1:20,000 dilution in TBS-T with 5% (w/v) milk. Immune complexes were detected using the enhanced chemifluorescence (ECF) reagent in the imaging system Typhoon FLA 9000 (GE Healthcare, UK), following a new washing step with TBS-T. Total Lab TL 120 software (GE Healthcare, UK) was used to quantify the optical density of the bands and the results obtained were normalized to β-tubulin I, which was used as a protein loading control.

After protein quantification by the BCA protein assay or acetone precipitation, the samples obtained from primary control and BD monocytes were denatured with 5x concentrated sample buffer and heated for 5 min at 95 ºC. In the first case, all samples were diluted to obtain a protein concentration of 30 µg that was applied in the gel. In the second case, precipitated samples were directly applied in the gel.

The subsequent steps of the WB procedure were performed as described above.

Table 3. Source, purification, molecular weight (kDa) and dilutions of the primary and secondary antibodies used for Western blotting analysis.

Antibodies	Source	Purification	MW (kDa)	Dilution
Primary Antibodies				
Anti-ATF4	Rabbit	Monoclonal	49	1:1,000
Anti-pro Caspase1+p10+p12	Rabbit	Monoclonal	45,42,35,12,10	1:1,000
Anti-CHOP	Mouse	Monoclonal	35	1:1,000
Anti-GRP78	Mouse	Monoclonal	78	1:1,000
Anti-IκBα	Rabbit	Polyclonal	39	1:1,000
Anti-IL-1β	Rabbit	Polyclonal	31	1:500
Anti-Mitofusin 2	Mouse	Monoclonal	75	1:1,000
Anti-NLRP3	Rabbit	Monoclonal	117	1:1,000
Anti-Sigma-1R	Goat	Polyclonal	25	1:500
Anti-XBP1s	Mouse	Monoclonal	54	1:500
Anti-p-IKBα	Mouse	Monoclonal	40	1:1,000
Anti-β-Tubulin I	Mouse	Monoclonal	55	1:20,000
Secondary Antibodies				
Anti-goat	Rabbit	_	_	1:20,000
Anti-mouse	Goat	_	_	1:20,000
Anti-rabbit	Goat	_	_	1:20,000

3.3.5. Detection of IL-1β levels in the cell supernatant

An ELISA kit was used to quantify the production of IL-1β induced by ER stress in THP-1 culture supernatants, according to the manufacturer's instructions. For that, human THP-1 monocytes were plated in 12-well plates at a density of 1.2 x 10^6 cells/well in a final volume of 1.5 mL. Once plated, cells were pre-treated with LPS for 24 h, prior to treatment with tunicamycin (5, 10 μg/mL) or thapsigargin (5, 10 μM) for 8 h in the presence or absence of ATP (5 mM) for 30 min or only treated with tunicamycin (5, 10 μg/mL) or thapsigargin (5, 10 μM) for 8 or 32 h. After incubation, cells were centrifuged at 400 g for 5 min and the supernatants were collected and stored at -80° C.

Defrosted samples and all kit reagents were equilibrated at RT before analysis. Thereafter, the plate was washed 4 times with 300 μL/well of Wash Buffer 1x (previously diluted 20x in deionized water). Then, 50 μL of Assay Buffer D was added to each well, which was followed by the addition of 50 μL of standard or samples. The human IL-1β standards (2 to 125 pg/mL) were prepared from an IL-1β standard stock solution (125 pg/mL) and were submitted to the same procedure as the samples. Samples without priming were diluted 10 x while LPS-primed samples were diluted 18 x. The sealed plate was incubated during 2 h, at RT with shaking at 200 rpm. After the incubation time, the plate was washed 4 times with Wash Buffer 1x (all subsequent washes were performed similarly) and 100 μL of human IL-1β Detection Antibody solution was added to each well. The plate was again incubated for 1 h, at RT, with shaking. After additional 4 washes, 100 μL of Avidin-HRP D solution were added to the plate, which was then sealed and incubated for 30 min with shaking, at RT. In the last set of washes, wells were soaked in Wash Buffer 1x for 45 seconds to minimize background. After addition of 100 μL of Substrate Solution F, the plate was incubated for 20 min in the dark. Wells containing human IL-1β turned blue in color with an intensity proportional to its concentration. Finally, 100 μL of Stop Solution was added per well and the color changed from blue to yellow. Absorbance values were measured in a standard spectrophotometer Synergy HT Multi Detection Microplate Reader (BioTek Instruments, USA) set to 450 nm and 570 nm wavelengths. Similarly to what was described for the BCA protein assay (section 3.3.3.), the IL-1β levels present in each sample were calculated by linear regression using the graph of IL-1β present in standards (X-axis)

versus absorbance (Y-axis).

To determine the production of IL-1β induced by tunicamycin or thapsigargin treatment in monocytes isolated from healthy donors or BD patients, the ELISA kit for human IL-1β was performed as described above. For this purpose, primary monocytes were plated in a 96-well plate at a density of 0.12×10^6 cells/well in a final volume of 0.15 mL. Once plated, monocytes were pre-treated with LPS (priming) for 24 h and then treated with tunicamycin (5, 10 µg/mL) or thapsigargin (5, 10 µM) for 8 h or were treated with the same doses of the ER stressors for 8 h or 32 h (no priming).

3.3.6. Detection of mitochondrial ROS production

MitoSOX Red is a fluorescent probe used to measure the superoxide production in the mitochondrial matrix (Polster et al, 2014). In this work, MitoSOX was used to determine the production of superoxide within mitochondria induced by tunicamycin or thapsigargin. For that, human THP-1 monocytes were plated in 12-well plates at a density of 1.2×10^6 cells/well in a final volume of 1.5 mL and treated with tunicamycin (5, 10 µg/mL) or thapsigargin (5, 10 µM) for 8 h. After ER stress induction, cells were washed with PBS to remove the medium and centrifuged at 300 g for 5 min. Afterwards, cells were resuspended in 1 mL of Hanks' Balanced Salt Solution (HBSS) containing 5 mM MitoSOX and 0.5 µg/mL Hoechst 33342 (nuclei marker due to DNA staining) and subsequently incubated for 10 min at 37 ºC. After this incubation period, cells were washed again with PBS and resuspended in 1mL HBSS free of supplements. Finally, the fluorescent signal present in the cell suspension (200 µL) was monitored using an Observer Z.1 fluorescence microscope (Zeiss, Germany). Images were captured using the Apochromat 63x/1.40 Oil DIC M27 objective.

3.3.7. Determination of mitochondrial membrane potential

Tetramethylrhodamine ethyl ester (TMRE) is a red-orange fluorescent probe used to label active mitochondria. In the case of depolarized mitochondria, the membrane potential decreases and the organelles are unable to sequester TMRE (Tomas-Gamasa et al, 2016). Therefore, TMRE was used to evaluate alterations in mitochondrial membrane potential upon tunicamy-

cin or thapsigargin treatment.

For this purpose, human THP-1 monocytes were plated in a 48-well plate at a density of 0.3 x 10^6 cells/well in a final volume of 300 µL and treated with tunicamycin (5, 10 µg/mL) or thapsigargin (5, 10 µM) for 8 h. A potent uncoupler of oxidative phosphorylation in mitochondria, carbonyl cyanide p- trifluoromethoxyphenylhydrazone (FCCP), which disrupts ATP synthesis and depolarizes mitochondrial membrane, was used as a positive control (10 min incubation, 50 µM). After treatments, cells were incubated with 1 µM TMRE for 30 min at 37 ºC. Then, cells were washed with PBS containing 0.2 % (w/v) BSA and pelleted by centrifuged at 300 g for 5 min. Finally, they were suspended in 200 µL PBS containing 0.2 % (w/v) BSA and the fluorescence was monitored with a standard spectrophotometer Synergy HT Multi Detection Microplate Reader (BioTek Instruments, USA) set to 549 nm excitation and 575 nm emission wavelengths.

3.3.8. Total lipid extraction

Lipid extraction was performed according to the Bligh & Dyer method (Bligh & Dyer, 1959) with modifications. For this assay, human THP-1 monocytes were cultured in T75 flasks at a density of 1 x10^6 cells/mL in a final volume of 10 mL and treated with tunicamycin (5, 10 µg/mL) or thapsigargin (5, 10 µM) for 8 h. Cells were pelleted by centrifugation at 300 g for 5 min and stored at -80 ºC until further use.

Once equilibrated at RT, the pellets were solubilized in 1 mL of ultra-pure water (MiliQ H2O), then 3.75 mL of a chloroform/methanol 1:2 (v/v) mixture were added and incubated on ice for 30 min upon strong vortex. An additional volume of 1.25 mL chloroform was added followed by addition of 1.25 mL MiliQ H2O; in all steps the mixtures were strongly vortexed. Samples were centrifuged at 252 g for 5 min at RT to obtain a two-phase system: an aqueous top phase and an organic bottom phase. The total lipid extract was recovered from the organic phase (bottom phase) into a new tube. A second extraction was performed by the addition of 1.88 mL chloroform to the aqueous phase and subsequent vortex for approximately 1 min. After centrifugation under the above conditions (252 g, 5 min, RT), the organic phase was added to the first extract. An extra extraction was followed by the addition of 1 mL of MiliQ

H$_2$O followed by centrifugation (252 g, 5 min, RT) to promote the clearance of the aqueous phase because the samples exhibited a dense protein fraction. Chloroform evaporation was done with a nitrogen stream. After drying, total lipid extracts were resuspended twice in 300 µL chloroform and vortexed strongly. The final volume was collected to vials and dried under a nitrogen flow. The samples were stored at -20 ºC for subsequent analysis. All solvents used in this protocol were HPLC grade.

3.3.9. Phosphate quantification

After lipid extraction, the total amount of phospholipids was quantified with the phosphate assay, as previously described by Bartlett and Lewis (Bartlett & Lewis, 1970) with modifications.

Lipid extracts were dissolved in 300 µL of chloroform, then 10 µL of each sample were dried under a nitrogen stream and then 125 µL of concentrated perchloric acid (70% w/v) was added. After incubation for 1 h at 180°C in the heating block (Stuart, UK), 825 µL of MiliQ H$_2$O, 125 µL of 2.5% (w/v) ammonium molybdate and 125 µL of 10% (w/v) ascorbic acid were added to all samples. The reaction mixture was homogenized in a vortex after each addition, which was followed by incubation for 10 min at 100°C in a Precisterm water bath (P Selecta, Spain). Then, samples were placed on cold water. Phosphate standards (0.1 to 2 µg) were prepared from a NaH$_2$PO$_4$.2H$_2$O stock solution (100 µg/mL) and were submitted to the same procedure described for samples with exception of the heating block step. Finally, 200 µL of each standard and sample were added to a 96-well plate and the absorbance values were measured in a spectrophotometer Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, USA) set to 797 nm. The amount of phosphate present in each sample was calculated by linear regression using the graph that correlates amount of phosphate levels present in standards (X-axis) and absorbance values (Y-axis). For lipids extracted from samples, the amount of phospholipid (µg) was calculated by the determined phosphate content, since each phospholipid has a phosphate group in its structure. All solvents used in this protocol were HPLC grade.

3.3.10. Phospholipid separation by thin layer chromatography

Thin layer chromatography (TLC) was used to separate several phospholipid classes present in total lipid extracts in order to evaluate alterations in THP-1 monocytes' lipid profile triggered by tunicamycin- or thapsigargin-induced ER stress.

Prior to separation, the TLC silica gel plate was washed in a chloroform/methanol mixture (1:1, v/v) and left in the safety hood for 15 min. Then, the plate was sprayed with 2.3% (w/v) boric acid and dried in an oven at 100 °C during 15 min. After loading total lipid extract (30 µg) dissolved in chloroform on the TLC plate, it was eluted with a mixture of chloroform/ethanol/water/triethylamine (30:35:7:35, v/v/v/v) during 3 h. Lipid spots on the silica plate were revealed by spraying the plate with a 50 µg/mL primuline solution dissolved in an acetone/water 80:20 mixture and visualized with an UV lamp (k = 254 nm). Identification of phospholipid classes was carried out by using phospholipid standards: sphingomyelin (SM), phosphatidylcholine (PC), cardiolipin (CL), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), lysophosphatidylcholine (LPC), phosphatidylglycerol (PG), and phosphatidic acids (PA), which ran side by side in the TLC plate. Afterwards, spots coincident with the migration of standards were scraped into glass tubes and quantified by the phosphate assay, as described in the previous section. All solvents used in this protocol were HPLC grade.

3.3.11. Electron microscopy

Electron microscopy was used to evaluate potential morphological alterations under tunicamycin- or thapsigargin-induced ER stress conditions in human THP-1 monocytes. For this purpose, cells were plated in 6-well plates at a density of 2.4×10^6 cells/well in a final volume of 3 mL and were then treated with tunicamycin (5, 10 µg/mL) thapsigargin (5, 10 µM) for 8 h.

After treatments, cells were collected and centrifuged at 1008 g, for 5 min. The supernatant was discarded and the pelleted cells were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h. Cells were rinsed in the same buffer and post-fixation was performed using 1% (w/v) osmium tetroxide for 1 h. After rinsing with buffer, samples were dehydrated in a graded ethanol series (70-100%). Following embedding in 2% (w/v) molten

agar, samples were re-dehydrated in ethanol (30-100%), impregnated and included in Epoxy resin (Fluka Analytical, Germany). Ultrathin sections were mounted on copper grids and observations were carried out at 100 kV on Tecnai G2 Spirit BioTwin electron microscope (FEI, USA).

3.3.12. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using Student's unpaired t test with one-tailed p value when comparing two groups. One-way ANOVA with unpaired Dunnett's post-hoc test was used for multiple comparisons. In both cases a value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed with the Prism 7.0 GraphPad Software.

Chapter 4
Results

4.1. ER stress in human THP-1 monocytes

First, we established an *in vitro* model of ER stress in human THP-1 monocytes. The rationale was to select doses and time periods of incubation with well-known pharmacological ER stressors able to trigger the UPR without evoking relevant cytotoxicity in human monocytes.

4.1.1. Susceptibility of human monocytes under ER stress conditions

Susceptibility of human monocytes (THP-1 cell line) towards ER stress was assessed by the cell viability resazurin assay (**Figure 15**). Two classical pharmacological ER stress inducers were used: tunicamycin that impairs glycosylation of newly synthesized proteins within the ER thus leading to the disruption of protein folding (Heifetz et al, 1979), and thapsigargin, which is a specific inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) that impairs ER Ca^{2+} homeostasis (Zhao et al, 1996). Cell viability was evaluated 24 h after treatment of THP-1 monocytes with increasing concentrations of tunicamycin (0.025 to 10 µg/mL) or thapsigargin (0.5 to 10 µM).
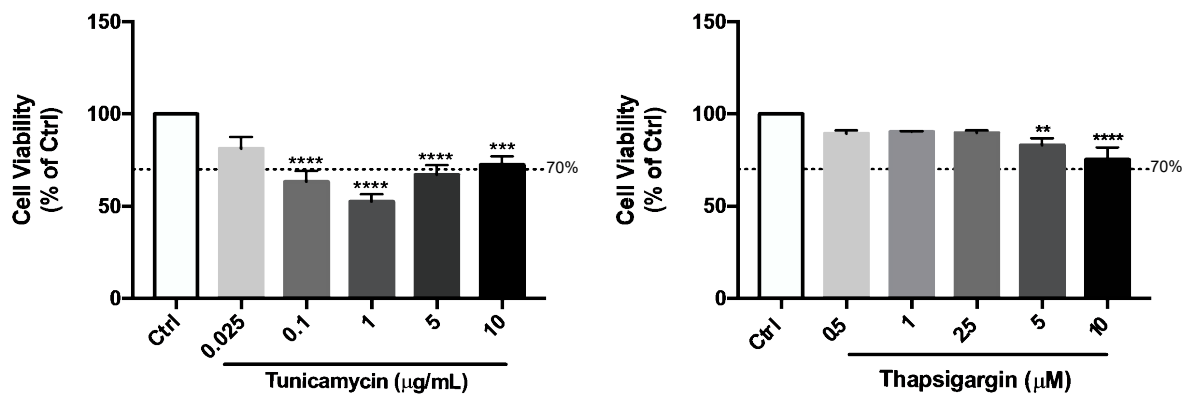
Figure 15. Susceptibility of human THP-1 monocytes towards ER stress. THP-1 cells were incubated with tunicamycin (0.025, 0.1, 1, 5 or 10 µg/mL) or thapsigargin (0.5, 1; 2.5, 5 or 10 µM) for 24 h. Cell's vulnerability towards tunicamycin- or thapsigargin-induced ER stress was assessed by the cell viability resazurin assay, as described in Methods section. Results represent the mean ± SEM of at least three independent experiments and were calculated relatively to control values (untreated cells). Statistical significance between control and treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test: ** $p<0.01$ *** $p<0.001$; **** $p<0.0001$.

In the case of tunicamycin, 0.025, 0.1 and 1 µg/mL decreased the number of viable THP-1 cells by approximately 20%, 40% and 50%, respectively. Both 5 and 10 µg/mL tunicamycin affected the monocyte's viability by approximately 30%.

Regarding the thapsigargin's dose-response curve, 0.5, 1 and 2.5 µM reduced cellular viability by 10%. At a 5 µM concentration, thapsigargin decreased the viability of THP-1 monocytes by almost 20% while 10 µM decreased the number of viable monocytes by approximately 25%.

Based on ISO 10993-5:2009 "Biological evaluation of medical devices-Part5: Tests for *in vitro* cytotoxicity", a reduction of cellular viability by more than 30% is considered a cytotoxic effect. Therefore, 5 and 10 µg/mL tunicamycin concentrations, which are in the border line (70% of viability), were chosen to pursue the experiments. For thapsigargin, 5 and 10 µM concentrations were selected for subsequent studies since they affected cellular viability by approximately 30% and don't exceeded the limit imposed by the aforementioned rule.

4.1.2. Expression of ER stress-induced UPR markers

UPR signaling pathways are activated in response to ER stress. Indeed, UPR is an extremely conserved process by which ER manages to restore cellular homeostasis and proteostasis, i.e. the balance between ER protein loading and folding (Decuypere et al, 2011; Deegan et al, 2013). Thus, to validate the ER stress model established in human THP-1 monocytes, protein levels of UPR markers were evaluated in tunicamycin- or thapsigargin-treated cells by western blotting (WB) analysis (**Figures 16 and 17**). Several UPR markers were investigated including: ATF4, an intermediate of the PERK pathway; XBP1s, an intermediate of the IRE1α pathway; GRP78, a major ER chaperone able to trigger UPR signaling upon ER stress with antiapoptotic properties; CHOP, a key transcription factor implicated in ER stress-induced apoptotic cell death (Bertolotti et al, 2000; Harding et al, 1999 Ron & Walter, 2007). The expression of the above UPR markers was assessed in a time-dependent manner (1-24 h) using two different tunicamycin (5 and 10 µg/mL) and thapsigargin (5 or 10 µM) concentrations.
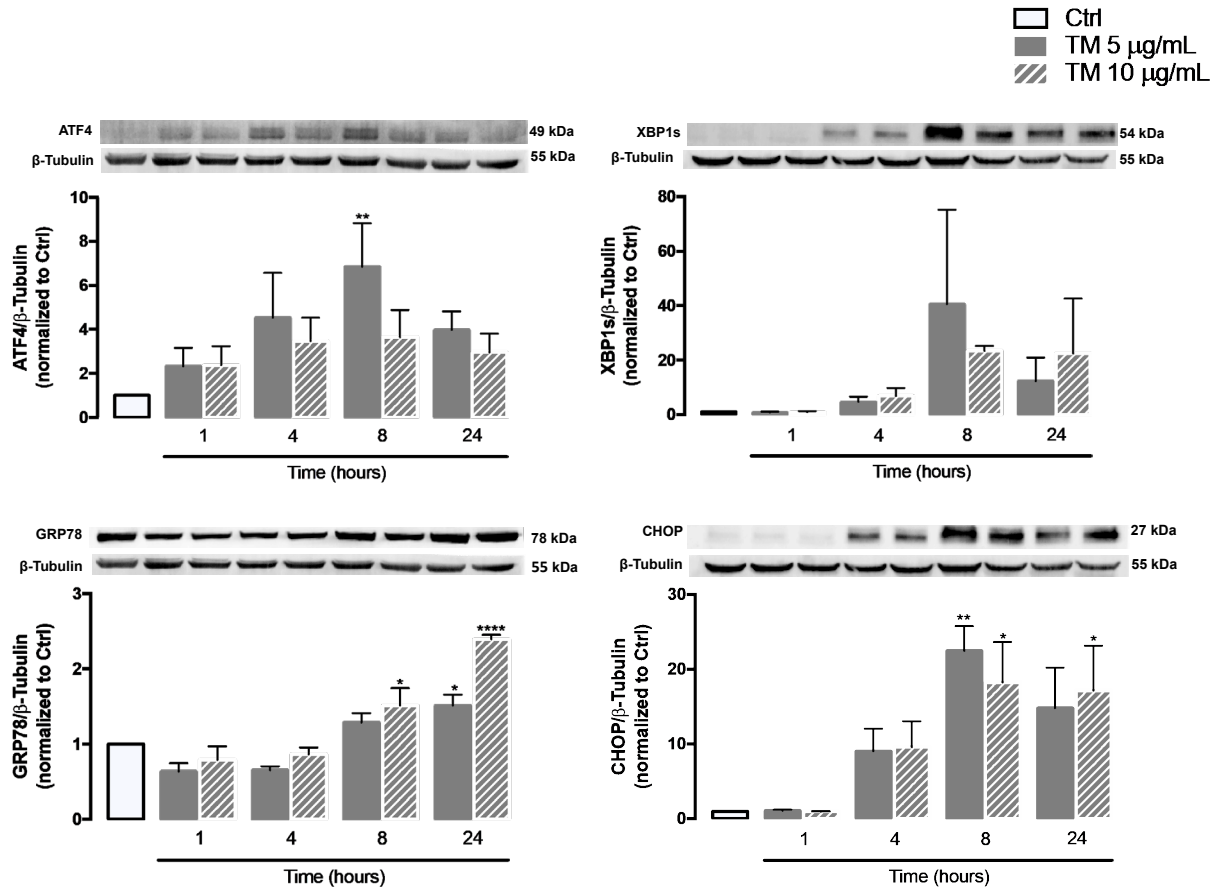
Figure 16. Protein levels of UPR markers under tunicamycin-induced ER stress in human THP-1 mono-cytes. Protein levels of ATF4, XBP1s, GRP78 and CHOP (ER stress-induced UPR markers) were quantified by WB in total cellular extracts obtained from the human monocytes' THP-1 cell line treated with 5 or 10 µg/mL tunicamycin during the indicated time periods (1-24 h). β-Tubulin I was used as a control for protein loading and to normalize the levels of the protein of interest. Results were calculated relatively to control values and represent the means ± SEM of at least three independent experiments (exception: XBP1s n=2). Statistical significance between control (untreated cells) and tunicamycin-treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test: *p <0.05; ** p<0.01; **** p<0.0001.

Protein levels of ATF4 and XBP1s increased in THP-1 cells upon incubation with tunicamycin when compared with controls, indicating the activation of the PERK- and IRE1α-mediated UPR pathways, respectively. The increase of ATF4 levels occurred in a time-dependent manner, with a statistically significant rise observed after 8 h of tunicamycin treatment, whereas XBP1s levels only demonstrated a tendency to increase at later time points (8 and 24 h). In addition, levels of the ER-resident chaperone GRP78 were significantly higher in tunicamycin-treated cells, occurring a time- and dose-dependent increase, with the peak at 24 h. Finally, levels of pro-apoptotic CHOP increased after tunicamycin exposure that was more pronounced after 8 h.
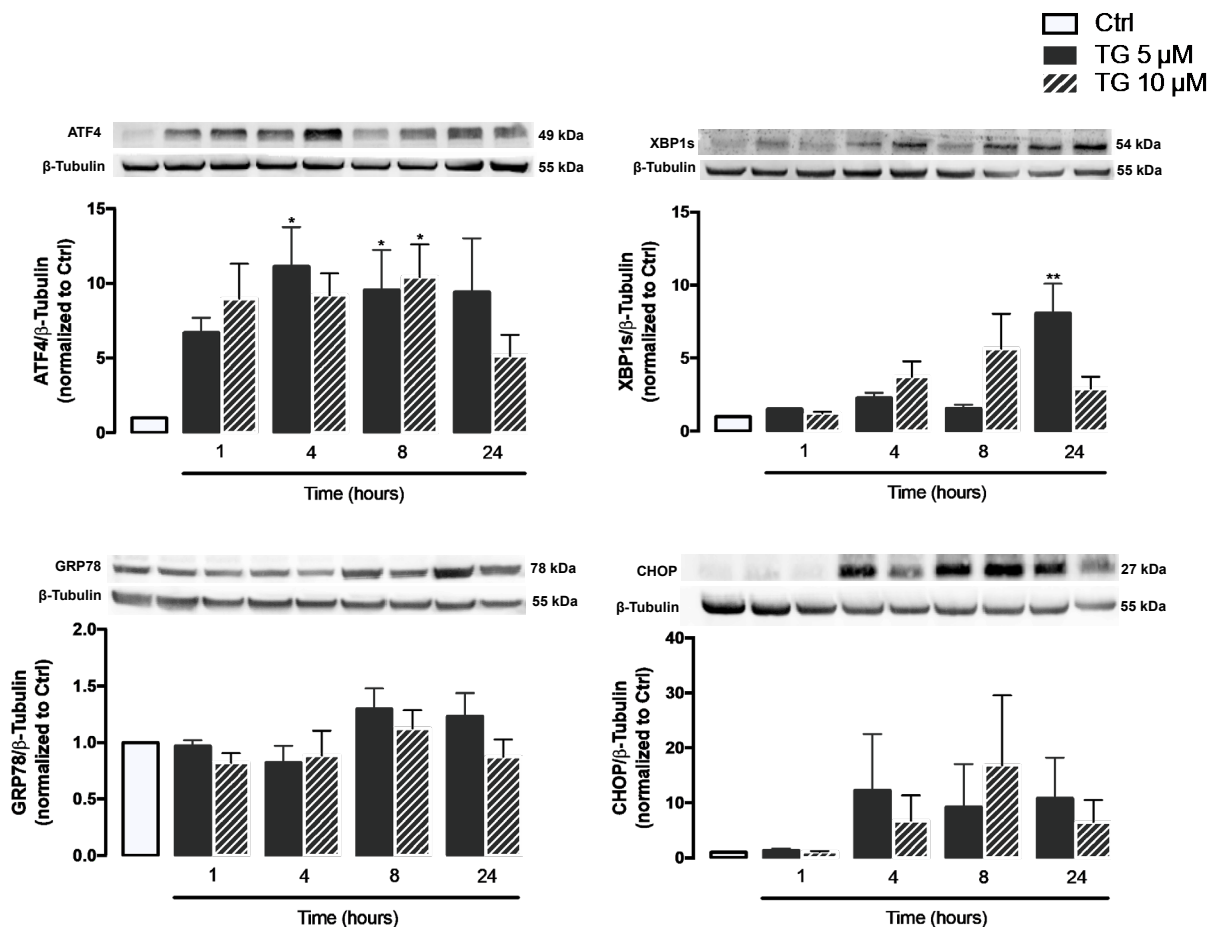
Figure 17. Protein levels of UPR markers under thapsigargin-induced ER stress in human THP-1 monocytes. Protein levels of ATF4, XBP1s, GRP78 and CHOP (ER stress-induced UPR markers) were quantified by WB in total cellular extracts obtained from the human monocytes' THP-1 cell line treated with 5 or 10 µM thapsigargin during 1-24 h. β-Tubulin I was used as a control for protein loading and to normalize the levels of the protein of interest. Results were calculated relatively to control values and represent the means ± SEM of at least three independent experiments. Statistical significance between control (untreated cells) and thapsigargin-treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test: *p<0.05; ** p<0.01.

As expected, both ATF4 and XBP1s protein levels were significantly increased upon thapsigargin exposure, demonstrating the activation of the PERK- and IRE1α-mediated UPR pathways, respectively. Furthermore, the protein levels of both GRP78 and CHOP also increased after treatment with thapsigargin during 4 and 24 h, further supporting that ER stress is induced in THP-1 monocytes by thapsigargin.

In conclusion, both tunicamycin and thapsigargin were demonstrated to be suitable pharmacological approaches to induce the UPR and establish an ER stress model in the human THP-1 monocytic cell line.

4.2. NLRP3 inflammasome activation in THP-1 monocytes exposed to ER stress

NLRP3 inflammasomes are multiprotein complexes formed by three components: NLRP3 protein, ASC and pro-caspase-1. After assembly of NLRP3 and ASC components, pro-caspase-1 is subsequently recruited to the platform, leading to the formation of the NLRP3 inflammasome, which induces the autocatalytic cleavage of caspase-1, processing of the cytokine precursor pro-IL-1β and, finally, IL-1β secretion (Guo et al, 2015; Hanamsagar et al, 2012; He et al, 2016; Walsh et al, 2014). Therefore, to evaluate NLRP3 inflammasome activation in THP-1 monocytes exposed to ER stress, the IL-1β levels (**Figure 18**), as well as the expression of NLRP3, pro-caspase-1 and pro-IL-1β, were assessed after cells treatment with tunicamycin or thapsigargin (**Figures 19 and 20**).

4.2.1. Levels of IL-1β in THP-1 monocytes under ER stress conditions

First, IL-1β levels were quantified as a readout of NLRP3 inflammasome activation in supernatants from cultured THP-1 cells treated with the ER stressors tunicamycin or thapsigargin, using an ELISA kit. It was observed that ER stress *per se* was not able to activate the NLRP3 and release IL-1β in human monocytes (data not shown). Since NLRP3 inflammasome activation is described as a two-step process that requires two signals (Guo et al, 2015; Hanamsagar et al, 2012; He et al, 2016; Walsh et al, 2014), THP-1 monocytes were primed with LPS (potent pro-inflammatory bacterial endotoxin) before ER stress induction. As a positive control, ATP (2nd signal) was administrated after pre-incubation with LPS (1st signal) since LPS plus ATP has been pointed out as a canonical trigger for NLRP3 inflammasome activation (Zha et al, 2016).

The release of IL-1β increased approximately 530-fold after treatment with the canonical activators of the NLRP3 inflammasome LPS plus ATP, relatively to the control condition (untreated cells). LPS alone increased IL-1β secretion by about 200-fold relatively to the control condition, and a similar increase was observed when IL-1β levels were compared in the supernatants from cells incubated with LPS and vehicle or with vehicle alone.
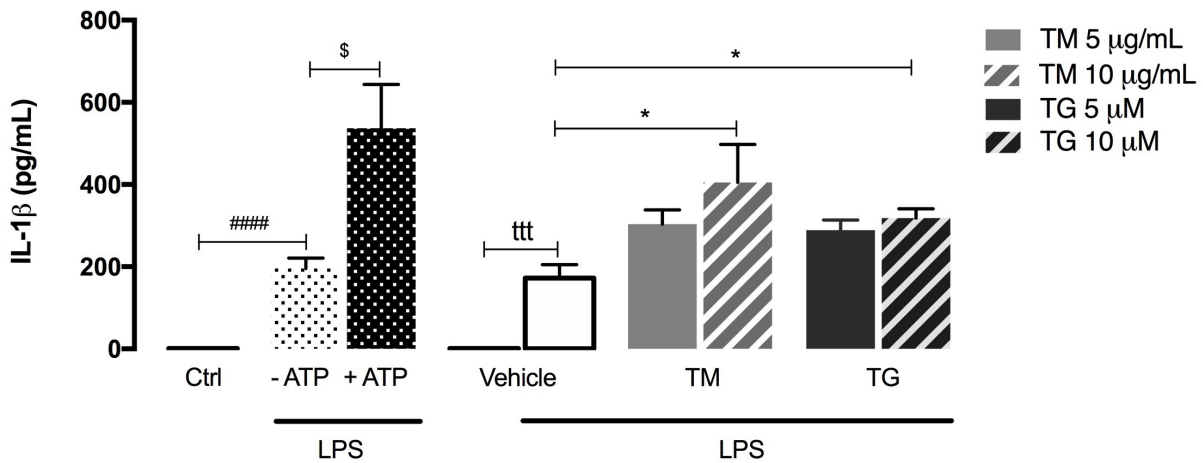
1β levels were determined using an ELISA kit in cell culture supernatants of human THP-1 monocytes treated with 1 µg/mL LPS alone (24 h), or pre-incubated with LPS and then treated with 5 µM ATP (30 min), tunicamycin (5 or 10 µg/mL) or thapsigargin (5 or 10 µM) for 8 h. Results represent the means ± SEM of at least three independent experiments. Statistical analysis: t-test was used for LPS-ATP and Ctrl (####$p<0.0001$), LPS+ATP and LPS-ATP ($\$p<0.05$), LPS+vehicle and vehicle (ttt$p<0.001$) comparisons, and one-way ANOVA with Dunnett's post-hoc test (*$p<0.05$; compared to LPS+vehicle) was used for multiple comparisons.

Interestingly, after an initial priming with LPS, ER stress activated the NLRP3 inflammasome, since secreted IL-1β levels increased upon tunicamycin and thapsigargin treatment, which occurred in a dose-dependent manner. Indeed, THP-1 monocytes incubated with LPS plus 10 µg/mL tunicamycin or with LPS plus 10 µM thapsigargin significantly increased the release of IL-1β (about 230-fold or 150-fold, respectively) when compared to monocytes treated with LPS and vehicle.

These findings strongly suggest that ER stress does not work as signal 1 (priming step) in THP-1 monocytes but can efficiently act as signal 2 (activation step) for NLRP3 inflammasome assembly, i.e., it may promote pro-IL-1β processing upregulated by LPS treatment.

4.2.2. Expression of the inflammasome-related proteins NLRP3, pro-caspase-1 and pro-IL-1β in THP-1 monocytes under ER stress conditions

In addition to quantification of secreted IL- 1β, protein levels of inflammasome components, namely NLRP3, pro-caspase-1, and pro-IL-1β, were analyzed by WB in THP-1 monocytes treated with tunicamycin (**Figure 19**) or thapsigargin (**Figure 20**) for 8 h.
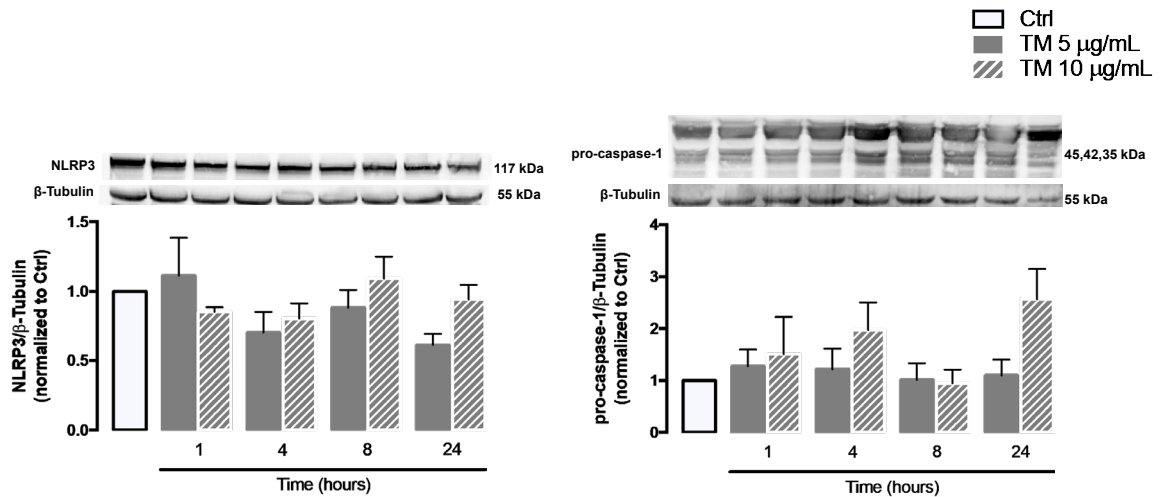
Figure 19. NLRP3 and pro-caspase-1 protein levels under tunicamycin-induced ER stress conditions in human THP-1 monocytes. Protein levels of NLRP3 and pro-caspase-1 were quantified by WB in total cellular extracts obtained after treatment of THP-1 cells with tunicamycin (5 or 10 µg/mL), during the indicated time periods (1-24 h). β-Tubulin I was used as a control for protein loading and NLRP3 and pro-caspase-1 levels were normalized to β-Tubulin. Results were calculated relatively to control values and represent the means ± SEM of at least three independent experiments. Statistical significance between control (untreated cells) and tunicamycin-treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test.

Tunicamycin was not able to modulate the expression of NLRP3 or pro-caspase-1 in THP-1 monocytes since the levels of these proteins were not significantly different from those determined in untreated cells. The protein levels of pro-IL-1β were also analyzed in total cellular extracts obtained from tunicamycin-treated THP-1 cells, however, it was not possible to detect any band corresponding to the pro-IL-1β molecular weight (data not shown).

Regarding thapsigargin (**Figure 20**), this ER stress inducer negatively modulated the expression of NLRP3 and pro-caspase-1 in THP-1 monocytes, since the levels of these proteins decreased approximately 50% in comparison with untreated cells. NLRP3 levels decreased at 4 and 8 h of thapsigargin treatment and a slight recovery was observed at 24 h, whereas pro-caspase-1 levels were significantly reduced at 8 and 24 h. Pro-IL-1β protein levels increased over time in THP-1 cells upon thapsigargin-induced ER stress, reaching a statistically significant increase after 24 in the presence of 5 µM thapsigargin.

Figure 20. NLRP3, pro-caspase-1 and pro-IL-1β protein levels under thapsigargin-induced ER stress conditions in human THP-1 monocytes. Protein levels of NLRP3, pro-caspase-1 and pro-IL-1β were quantified by WB in total cellular extracts obtained after THP-1 cells treatment with thapsigargin (5 or 10 µM), during the indicated time periods (1-24 h).β-Tubulin I was used as a control for protein loading and to normalize the levels of the protein of interest. NLRP3 and pro-caspase-1 protein levels were calculated relatively to control values. Results represent the means ± SEM of at least three independent experiments. Statistical significance between control (untreated cells) and thapsigargin-treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test: *p<0.05; ** p<0.01; **** p<0.0001.

Together, these results support the hypothesis that tunicamycin and thapsigargin don't function as signal 1 for NLRP3 inflammasome activation in THP-1 monocytic cell line because they don't upregulate the expression of the NLRP3 inflammasome-related proteins, namely NLRP3 and pro-caspase-1. Furthermore, diminished levels of pro-caspase-1 predict a decrease in its active form that may explain the observed increase of pro-IL-1β, however further studies are required to determine caspase-1 activation.

4.3. Activity of the transcription factor NF-κB in human THP-1 monocytes exposed to ER stress

The first signal (priming step) induces the upregulation of NLRP3 inflammasome components, such as NLPR3 and pro-IL-1β, to ensure its assembly. For that, the priming signal activates transcription factors, mainly NF-κB (Bauernfeind et al, 2009; Franchi et al, 2009). After activation, NF-κB is translocated to the nucleus leading to the transcription of multiple genes involved in innate immune responses. The activity of this ubiquitous transcription factor is regulated by an inhibitor protein, IKBα, which sequesters NF-κB in the cytoplasm thus preventing its activation. In response to extracellular signals, IKBα is phosphorylated and selectively degraded in the proteasome, leading to NF-κB activation (Kretz-Remy et al, 1996; Miyamoto et al, 1994). Therefore, to investigate NF-κB activation in human THP-1 monocytes exposed to ER stress, protein levels of both phospho-IKBα and IKBα were determined in cells treated with tunicamycin (5 or 10 µg/mL) or thapsigargin (5 or 10 µM) during the indicated time periods (5-60 min).
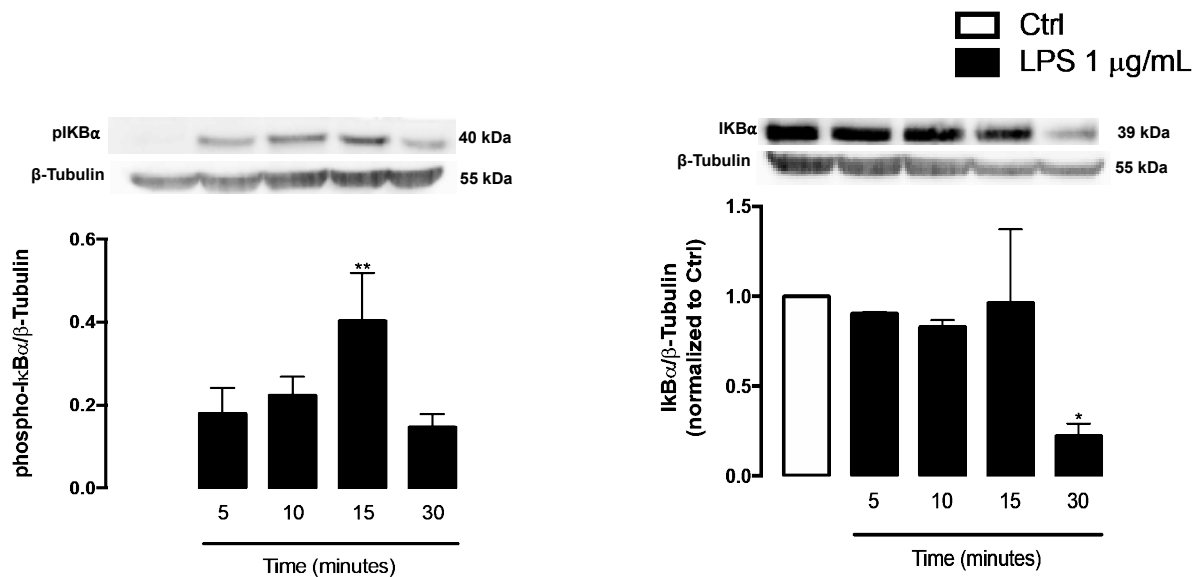
Figure 21. Phospho-IKBα and IKBα protein levels in LPS-treated human THP-1 monocytes. Protein levels of phospho-IKBα and IKBα were quantified by WB in total cellular extracts obtained after treatment of THP-1 cells with 1 µg/ml LPS during the indicated time periods (5-30 min). β-Tubulin I was used as a control for protein loading and to normalize the levels of the protein of interest. IKBα protein levels were calculated relatively to control values. Results represent the means ± SEM of at least three independent experiments. Statistical significance between control (untreated cells) and LPS-exposed cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test: *p<0.05; ** p<0.01.

Before determining the modulation of phospho-IKBα and IKBα protein levels in response to tunicamycin- or thapsigargin-induced ER stress in THP-1 monocytes (**Figures 22 and 23**), a time-dependent analysis of IKBα phosphorylation and subsequent degradation was performed by WB for 5-30 min using LPS as a positive control for NF-kB activation (**Figure 21**).

Protein levels of phospho-IKBα increased in response to LPS, with a statistically significant rise detected 15 min after cellular exposure. A significant decrease in protein levels of IKBα was observed 30 min upon LPS incubation. In conclusion, in THP-1 human monocytes submitted to pro-inflammatory conditions triggered by LPS, maximal IKBα phosphorylation and IKBα degradation occurred at 15 and 30 min, respectively.

Figure 22. Phospho-IKBα and IKBα protein levels under tunicamycin-induced ER stress conditions in human THP-1 monocytes. Protein levels of phospho-IKBα and IKBα were quantified by WB in total cellular extracts obtained from THP-1 cells treated with tunicamycin (5 or 10 µg/mL), during the indicated time periods (5-60 min). β-Tubulin I was used as a control for protein loading and to normalize the levels of the protein of interest. Results were calculated relatively to control values and represent the means ± SEM of at least three independent experiments. Statistical significance between control (untreated cells) and tunicamycin-treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test.

As observed in LPS-treated monocytes (**Figure 21**), phospho-IKBα protein levels increased under tunicamycin-induced ER stress conditions and IKBα protein levels decreased in a time-dependent manner, although the alterations didn't reach statistical significance.

Under thapsigargin-induced ER stress conditions (**Figure 23**), protein levels of phospho-IKBα increased whereas levels of IKBα decreased in a time-dependent manner in THP-1 monocytes. In cells treated with 5 µM thapsigargin for 60 min a statistically significant depletion of IKBα was found.

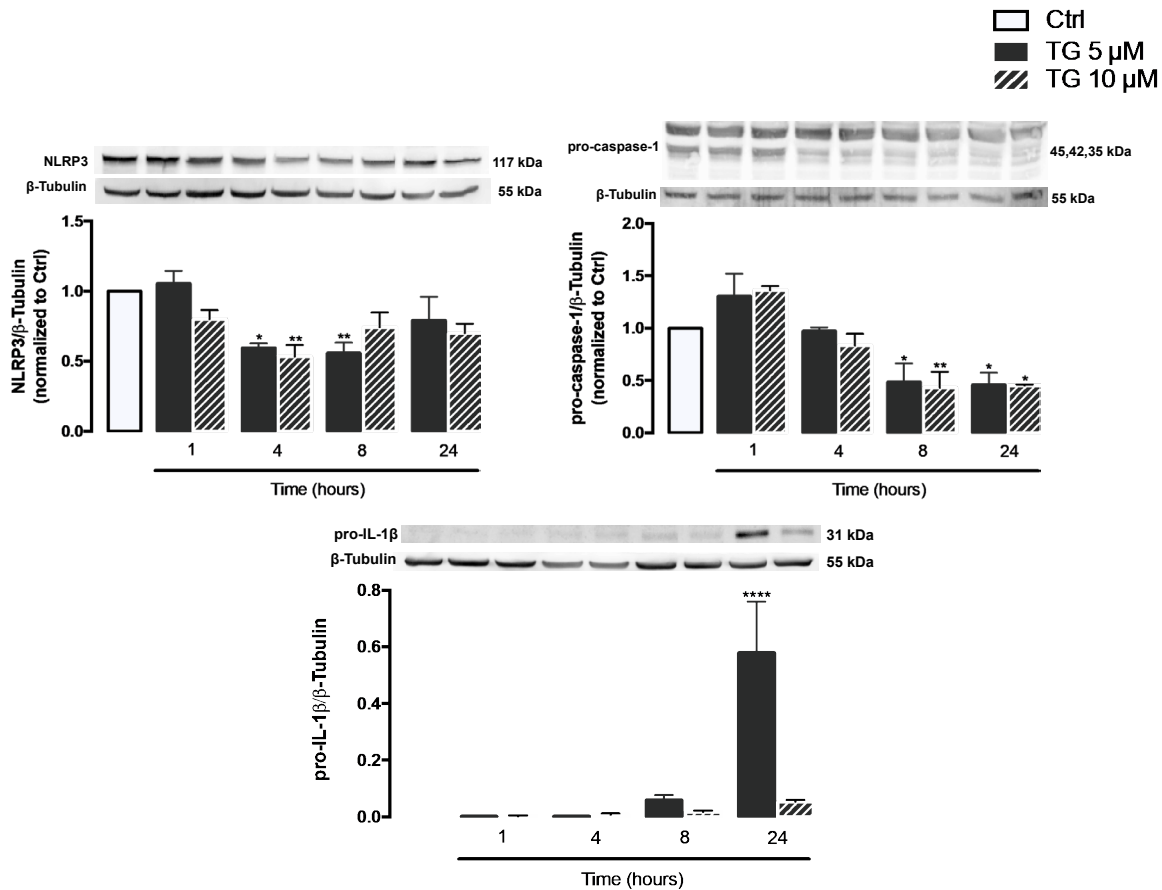**Figure 23. Phospho-IKBα and IKBα protein levels under thapsigargin-induced ER stress conditions in human THP-1 monocytes.** Protein levels of phospho-IKBα and IKBα were quantified by WB in total cellular extracts obtained from THP-1 cells treated with thapsigargin (5 or 10 μM) during the indicated time periods (5-60 min). β-Tubulin I was used as a control for protein loading and to normalize the levels of the protein of interest. Results were calculated relatively to control values and represent the means ± SEM of, at least, three independent experiments. Statistical significance between control (untreated cells) and thapsigargin-treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test: ** $p < 0.01$.

It can be concluded that both tunicamycin and thapsigargin modulate NF-κB activity, however, ER stress-induced NF-κB activation is not able to activate the NLRP3 inflammasome (**Figure 18**) since it doesn't up-regulate NLRP3 or pro-caspase-1 (**Figures 19 and 20**).

4.4. ER-mitochondria communication at MAMs during ER stress-induced NLRP3 activation in human THP-1 monocytes

After demonstrating that ER stress activates the NLRP3 inflammasome in human THP-1 monocytes, acting as activation signal (second step) for its assembly, we proposed to decipher the molecular mechanisms behind NLRP3 activation. For this purpose, MAM's dysfunction was evaluated as the link between ER stress and inflammasome activation in human THP-1 monocytes by investigation MAM's structural and functional alteration in ER stressed THP-1 monocytes.

4.4.1. MAM's composition during ER stress in human THP-1 monocytes

To evaluate the effect of ER stress on MAM's structure, protein levels of two of its components, Sigma-1R and Mitofusin 2, were assessed by WB in monocytes treated with tunicamycin (**Figure 24**) or thapsigargin (**Figure 25**).
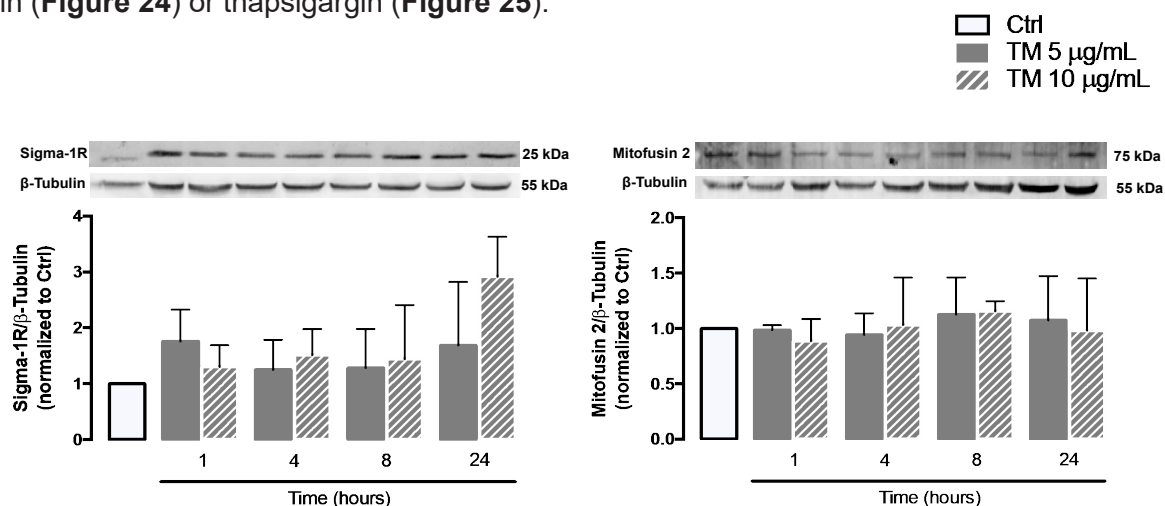
Figure 24. Sigma-1R and Mitofusin 2 levels under tunicamycin-induced ER stress conditions in human THP-1 monocytes. Protein levels of Sigma-1R and Mitofusin 2 were quantified by WB in total cellular extracts obtained after THP-1 cells treatment with tunicamycin (5 or 10 µg/mL), during the indicated time periods (1-24 h). β-Tubulin I was used as a control for protein loading and to normalize the levels of the protein of interest. Results were calculated relatively to control values and represent the means ± SEM of at least three independent experiments. Statistical significance between control (untreated cells) and tunicamycin-treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test.

Figure 25. Sigma-1R and Mitofusin 2 levels under thapsigargin-induced ER stress conditions in human THP-1 monocytes. Protein levels of Sigma-1R and Mitofusin 2 were quantified by WB in total cellular extracts obtained after THP-1 cells treatment with thapsigargin (5 or 10 µM) during the indicated time periods (1-24 h). β-Tubulin I was used as a control for protein loading and to normalize the levels of the protein of interest. Results were calculated relatively to control values and represent the means ± SEM of at least three independent experiments. Statistical significance between control (untreated cells) and thapsigargin-treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test: $*p<0.05$; $***p<0.001$; $****$ $p<0.0001$.

An increase in the MAMs-resident chaperone Sigma-1R was observed in THP-1 monocytes treated with tunicamycin for 1-24 h, although it didn't reach statistical significance. In addition, levels of the ER-mitochondria tethering protein Mitofusin 2 were unaffected by tunicamycin exposure since no differences were found between untreated and treated cells.

Tunicamycin and thapsigargin were shown to differently regulate MAM's structure. The expression of Sigma-1R was not regulated by thapsigargin treatment in THP-1 monocytes, however, Mitofusin 2 was highly up-regulated in thapsigargin-treated THP-1 cells. Indeed, after treatment with thapsigargin for 8 or 24 h, a significant increase of about 6- and 8-fold, respectively, was detected in Mitofusin 2 protein levels in comparison with untreated cells.

In conclusion, ER-mitochondria contacts at MAMs are altered upon ER stress in human THP-1 monocytes.

4.4.2. Lipid profile during ER stress in human THP-1 monocytes

Given that MAMs are lipid raft-like subdomains and the lipid metabolism, particularly the phospholipid synthesis, is one of its major functions (Area-Gomez et al, 2012; Missiroli et al, 2018), we evaluated how the lipid profile was modulated by ER stress in human THP-1 monocytes. Thus, upon ER stress induction, several phospholipid classes were evaluated by TLC (**Figure 26**).

Different relative abundances (%) were observed among the various classes of phospholipids in THP-1 cells. This preliminary study suggests that tunicamycin- and thapsigargin-induced ER stress may modulate the lipid profile of human monocytes affecting the relative abundance of several phospholipids, namely cardiolipins that are localized in the mitochondria and have been recently described as NLRP3 activators. However, cardiolipins were determined in total cell extracts and their levels and localization at the inner or outer mitochondrial membranes cannot be inferred.
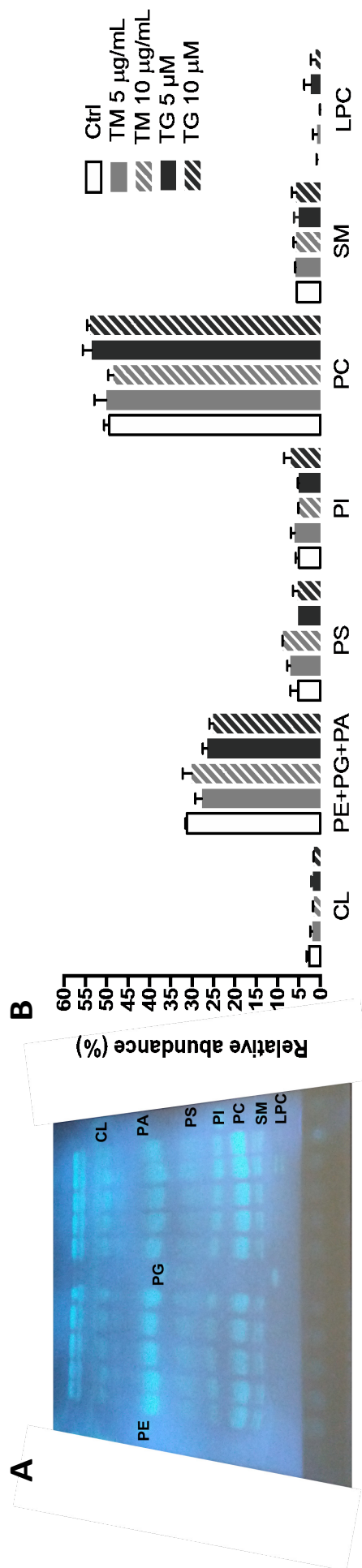
Figure 26. Phospholipid profile during ER stress in human THP-1 monocytes. Effect of the ER stress inducers tunicamycin and thapsigargin in the relative abundance of phospholipids (PLs) on THP-1 cells (A). PLs were extracted from untreated cells or cells treated, during 8h, with tunicamycin (5 or 10 µg/mL) or thapsigargin (5 µM or 10 µM) and were separated by thin layer chromatography and identified using PLs standards (B). The phosphate content of each PL spot was quantified and was expressed as a % of the phosphate content in the respective total lipid extract. CL, cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine. The mean values ± SEM of two independent experiments are shown.

4.4.3. MAM's function during ER stress in human THP-1 monocytes

To further explore MAM's deregulation as the link between ER stress and NLRP3 inflammasome activation, functional parameters mediated by ER-mitochondria contacts, namely mitochondrial ROS generation (**Figure 27**) and mitochondrial membrane potential (**Figure 28**) were evaluated in tunicamycin- and thapsigargin-treated THP-1 cells.
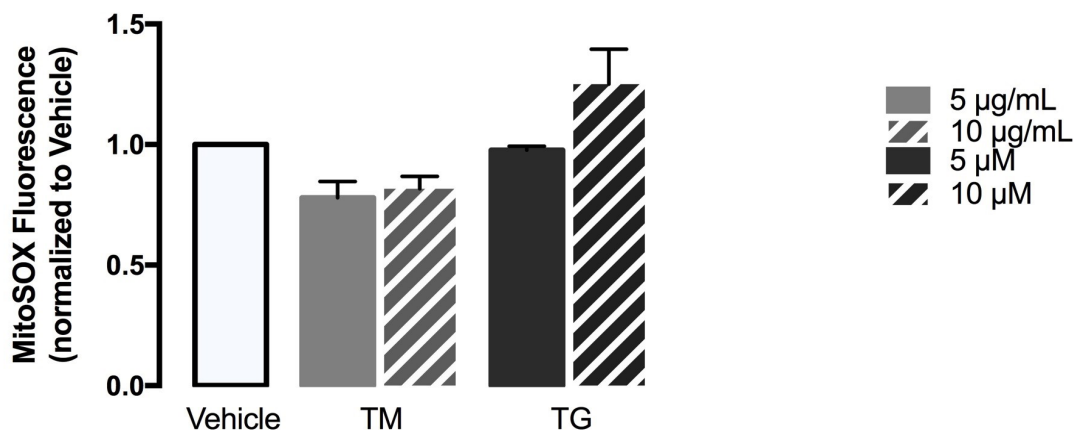
Figure 27. Mitochondrial ROS generation under ER stress conditions in human THP-1 monocytes. Production of mitochondrial superoxide was measured using the fluorescence probe MitoSOX in human THP-1 monocytes treated with tunicamycin (5 or 10 µg/mL) or thapsigargin (5 µM or 10 µM) for 8 h. Results were normalized to vehicle values and represent the means ± SEM of three independent experiments. Statistical significance between control cells (with vehicle) and cells treated with tunicamycin or thapsigargin was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test.
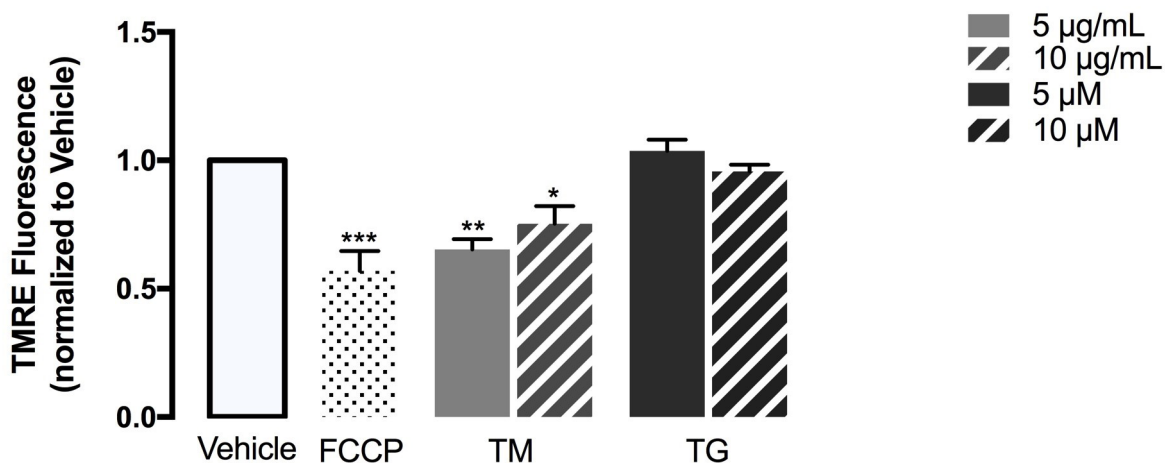
Figure 28. Mitochondrial membrane potential under ER stress conditions in human THP-1 monocytes. Alterations in mitochondrial membrane potential were measured using the TMRE fluorescent probe in human THP-1 monocytes treated with tunicamycin (5 or 10 µg/mL) or thapsigargin (5 µM or 10 µM), for 8 h. FCCP (50 µM) was used to depolarize the mitochondrial membrane (positive control). Results were calculated relatively to vehicle values and represent the means ± SEM of three independent experiments. Statistical significance between control cells (with vehicle) and cells treated with tunicamycin or thapsigargin was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test: *$p<0.05$; ** $p<0.01$; ***$p<0.001$.

In THP-1 monocytes treated with 5 or 10 μg/mL tunicamycin, a slight decrease of mitochondrial superoxide levels (**Figure 27**) was detected in comparison with controls (cells incubated with vehicle), whereas in monocytes treated with 10 μM thapsigargin an increase in mitochondrial superoxide levels was observed, which was not statistically significance.

It was also found that ER stress induced by 5 or 10 μg/mL tunicamycin in human THP-1 monocytes is associated with a depolarization of the mitochondrial membrane (**Figure 28**) since the TMRE fluorescence in these conditions was significantly decreased in comparison with that determined in cells incubated with the vehicle. The mitochondrial membrane potential of these cells was not affected by thapsigargin-induced ER stress, given that TMRE fluorescence in these conditions was similar to that observed in control conditions (cells with vehicle).

Based on these results, we may conclude that the activation of NLRP3 inflammasome induced by tunicamycin or thapsigargin in human THP-1 monocytes seems to be independent of mitochondrial superoxide production. Moreover, alterations on mitochondrial membrane potential may be behind the tunicamycin-induced NLRP3 inflammasome activation.

4.5. *Morphological alterations during ER stress-induced NLRP3 activation in human THP-1 monocytes*

To unveil the morphological alterations provoked by ER stress in human THP-1 monocytes, with a special focus on the ER and mitochondria morphology, cells were analyzed by electron microscopy after treatment with the two-well established ER stressors (**Figure 29**).

First, selected images show that there are no significant morphological changes between control and vehicle-treated THP-1 cells. In addition, when compared with untreated or vehicle-treated cells, several morphological alterations were observed in monocytes under ER stress, such as decreased cellular projections, which is particular evident after thapsigargin treatment (j), presence of dilated ER (g,i), increased number of tubular mitochondria (f, m) and, subsequently, an increase of both mitochondrial fission (l) and mitophagy (e). Moreover,

a significant increase of vacuoles (h,m) and autophagy-related structures (e,j), such as autophagosomes, were also detected in treated monocytes. The presence of more autophagic structures and darker dots resembling lysosomes suggest an enhancement of autophagy in monocytes under ER stress conditions.
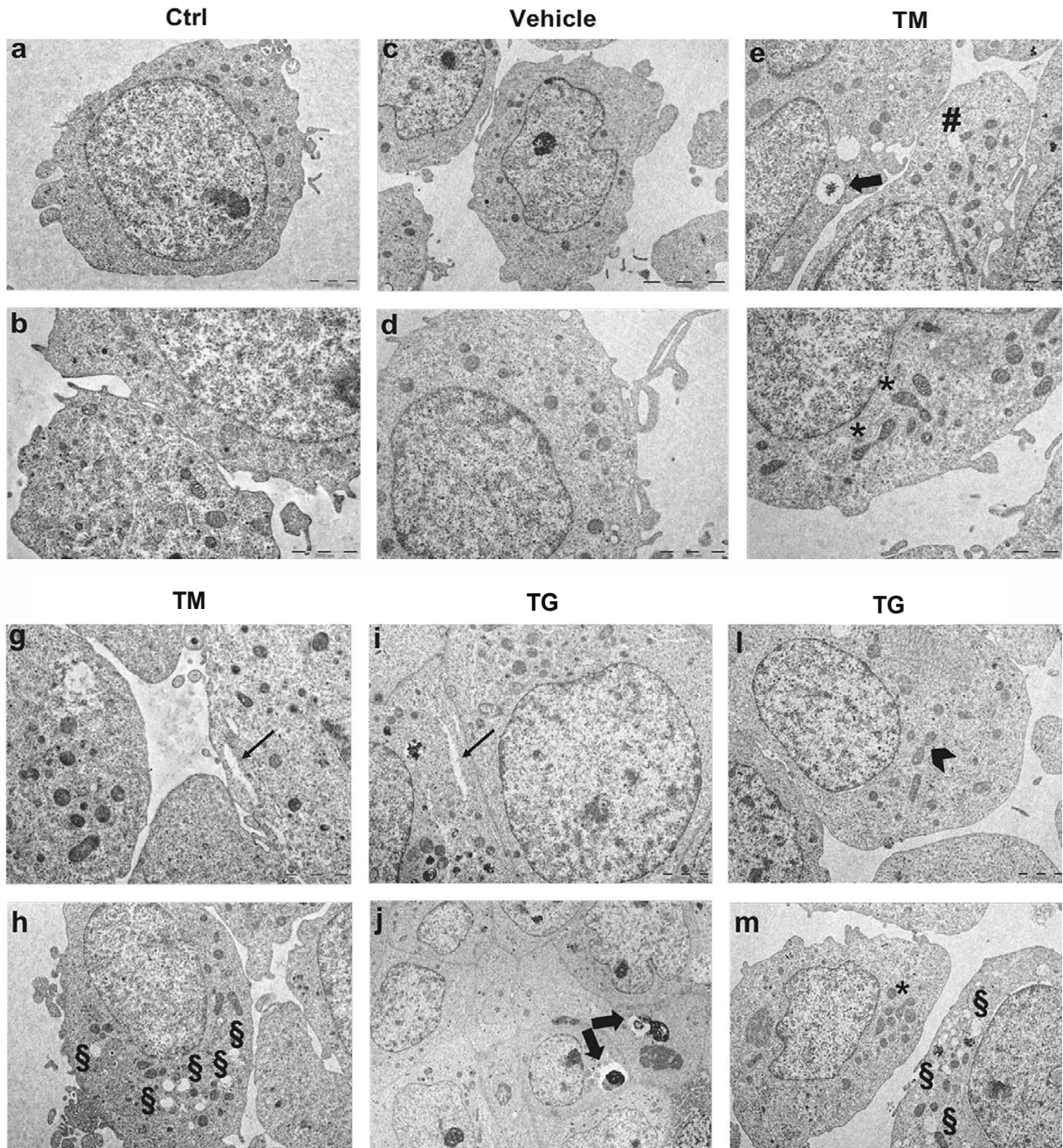
Figure 29. Morphological alterations under ER stress conditions in human THP-1 monocytes. Alterations in ER and mitochondria morphology due to ER stress were monitored by electron microscopy in human THP-1 monocytes treated with tunicamycin (5 or 10 µg/mL) or thapsigargin (5 µM or 10 µM) for 8 h. Control cells (a,b); vehicle-treated cells (c,d); tunicamycin-treated cells (e-h); and thapsigargin-treated cells (i-m). Dilated ER (fine arrows); mitochondria tubular (asterisks); mitochondrial fission (arrow head); mitophagy (cardinal); vacuoles (dollar sign) and autophagy-related structures (broad mushrooms).

Studies in primary monocytes derived from BD patients and matched controls

After investigating the role of ER stress in innate immunity focusing on NLRP3 inflammasome activation, and clarify the molecular mechanisms underlying ER stress-induced NLRP3 activation in the THP-1 cell line of human monocytes, we proposed to disclose how ER stress-inflammasome axis is modulated in a patient-derived cellular model of BD using primary monocytes isolated from patients and healthy control subjects.

It is important to emphasize that this is an exploratory pilot study with only 5 subjects from each group (controls versus BD patients), with great variability inter-donors. Furthermore, in consequence of the low yield of isolated monocytes (**Table 4**), most of the experiments performed do not contemplate the analysis of the 5 subjects per group under study. Therefore, in the future, is utmost important to increase the sample size.

Table 4. Yield of peripheral blood mononuclear cells (PBMCs) and monocytes isolation from healthy control subjects and BD patients (n=5, mean values ± SEM).

Isolation's yield	Healthy subjects	BD patients
PBMCS's yield	23.4 ± 1.4	24.2 ± 4.3
Monocyte's yield	4.96 ± 0.72	5.84 ± 0.79

4.6. ER stress-induced UPR in monocytes from healthy controls and BD patients

In a first approach, we investigated the ER stress-induced UPR in monocytes derived from healthy controls and BD patients. For this purpose, the expression levels of an early (ATF4) and a late (GRP78) UPR markers were evaluated by WB analysis in monocytes under basal conditions and upon treatment with the canonical ER stress inducers tunicamycin or thapsigargin (**Figure 30**).
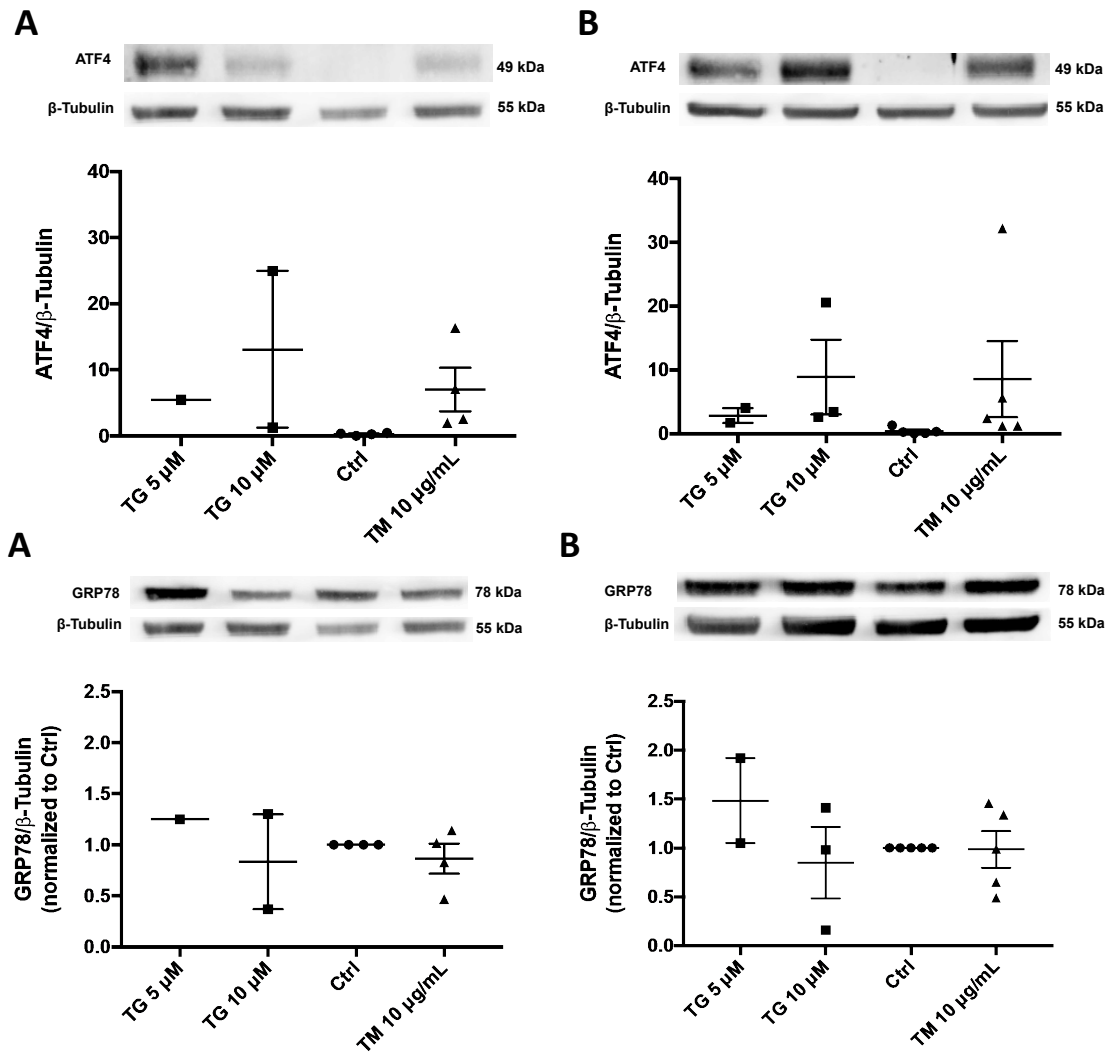
Figure 30. Protein levels of UPR markers under basal and ER stress conditions in monocytes from healthy controls and BD patients. Protein levels of ATF4 and GRP78 (ER stress-induced UPR markers) were quantified by WB in total cellular extracts obtained from monocytes derived from healthy controls (A) and BD patients (B) untreated or treated with 10 µg/mL tunicamycin (TM) or thapsigargin (TG) (5 or 10 µM) for 8 h. β-Tubulin I was used as a protein loading control to normalize the levels of the protein of interest. GRP78 protein levels were calculated relatively to control values. Results represent the means ± SEM of, at least, three independent experiments (with exception of 5 or 10 µM TG). Statistical significance between control (untreated cells) and treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test.

Although not statistically significant, an increase in the levels of ATF4 was observed in monocytes derived from healthy controls or BD patients under stimulated conditions, in comparison with those determined in basal conditions. As ATF4 is an intermediate of PERK UPR signaling pathway, this preliminary data validates the induction of ER stress in monocytes derived from healthy controls and BD patients.

Furthermore, a modest increase in GRP78 protein levels was determined in monocytes derived from healthy controls as well as in BD patients' monocytes treated with 5 µM thapsigargin relatively to basal conditions. However, it is important to note that the number of subjects tested on this condition is reduced (n=1 or 2, respectively). The GRP78 protein levels were not increased in control and BD treated with higher doses of ER stress inducers (10 µg/mL tunicamycin or 10 µM thapsigargin).

Based on the above preliminary data, it seems that monocytes from healthy control and BD monocytes respond to ER stress and that the response is not significantly different between monocytes from the two groups.

4.7. NLRP3 inflammasome activation in monocytes from healthy controls and BD patients under basal and ER stress conditions

In monocytes derived from healthy controls and BD patients, NLRP3 inflammasome activation was evaluated through quantification of secreted IL-1β levels under basal conditions and upon stimulation with the ER stressors tunicamycin and thapsigargin in the presence or absence of LPS (**Figures 31 and 32**), as well as by analysis of NLRP3 and pro-IL-1β expression (**Figure 33**).

4.7.1. Levels of IL-1β secreted in monocytes from healthy controls and BD patients

First, we evaluated the activation of the NLRP3 inflammasome in monocytes isolated from healthy controls and BD patients under basal conditions and upon exposure to tunicamycin- or thapsigargin-induced ER stress (without LPS priming) by measuring the IL-1β secretion as the main readout of inflammasome assembly and activation.
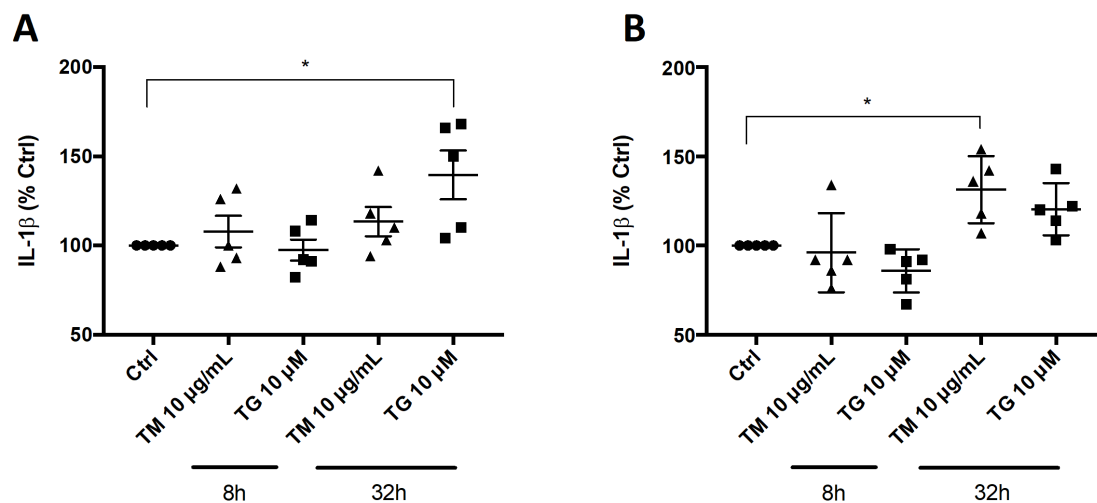
Figure 31. IL-1β levels in monocytes from healthy controls and BD patients under basal conditions and after exposure to ER stress. IL-1β levels in cell culture supernatants obtained from monocytes derived from healthy controls (A) and BD patients (B) treated with 10 µg/mL tunicamycin or 10 µM thapsigargin, during 8 or 32 h, were quantified using an ELISA kit and were compared with that determined in control and BD monocytes under basal conditions. Results were calculated relatively to control values (%) and represent the means ± SEM of five independent experiments. Statistical significance between control (untreated cells) and treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test. *$p < 0.05$.

In both healthy control- and BD patients-derived monocytes treated with the ER stress inducers during 8 h, the secretion of IL-1β was identical to that obtained in basal conditions.

In monocytes isolated from healthy controls exposed to ER stress conditions during 32 h, increased levels of secreted IL-1β were detected, with a statistically significant increase of approximately 40% determined in cells treated with 10 µM thapsigargin. In BD patients-derived monocytes, higher levels of secreted IL-1β were observed 32 h after ER stress induction. Particularly, an increase of about 30% of secreted IL-1β was measured in BD monocytes treated with 10 µg/mL tunicamycin.

Contrarily to what was observed in the THP-1 monocytic cell line, ER stress *per se* was shown to induce NLRP3 inflammasome activation after 32 h incubation with tunicamycin or thapsigargin in monocytes derived from healthy controls as well as from BD patients, suggesting that ER stress may act as signals 1 and 2 for NLRP3 activation. On the other hand, induction of ER stress during 8 h didn't activate the NLRP3 inflammasome, suggesting that ER stress is not able to work as signal 1 in short time periods.

4.7.2. Levels of IL-1β secreted in LPS-primed monocytes from healthy controls and BD patients

As the NLRP3 activation is described as a two-step process requiring two triggers (signal 1 plus signal 2) and given that ER stress does not work as signal 1 at 8 h, secreted IL-1β levels were determined in monocytes from healthy controls and BD patients using LPS pre-incubation as a priming signal prior to treatment with tunicamycin and thapsigargin (**Figure 32**). The rational of this set of experiments is to determine whether ER stress can function as signal 2 for the NLRP3 assembly.
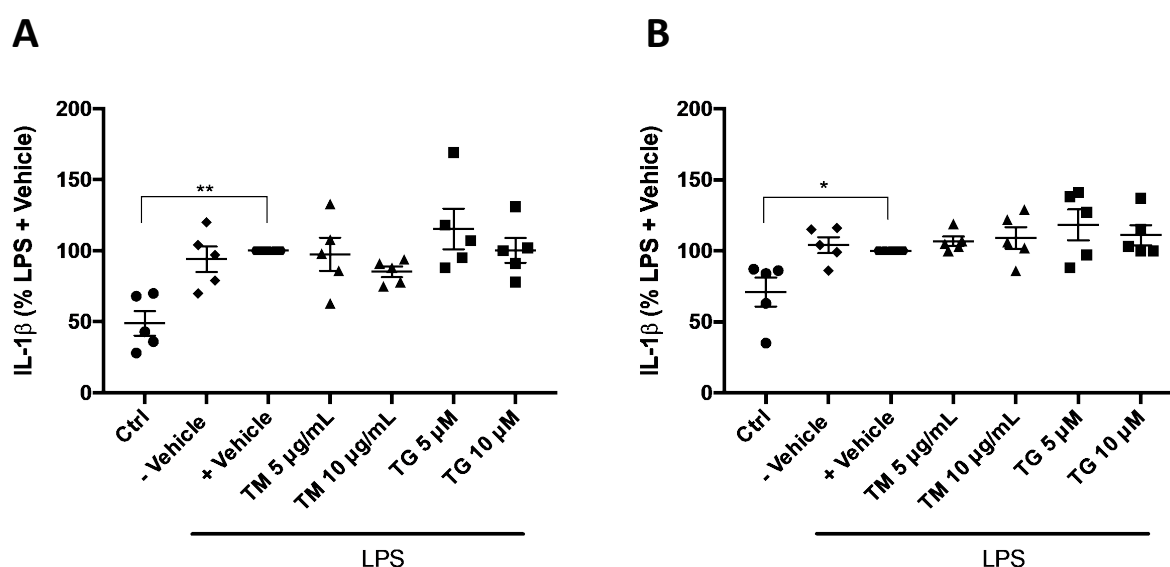
Figure 32. IL-1β levels in monocytes from healthy controls and BD patients exposed to LPS (priming) followed by ER stress. IL-1β levels in cell culture supernatants obtained from monocytes derived from healthy controls (A) and BD patients (B) cultured during 24 h with LPS plus 8 h of tunicamycin (5 or 10 µg/mL) or thapsigargin (5 or 10 µM) were quantified using an ELISA kit. Results were calculated relatively to values (%) determined in the LPS+vehicle condition and represent the means ± SEM of five independent experiments. Statistical significance between (LPS+vehicle)-treated cells and the remaining conditions was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test. *p<0.05; ** p<0.01.

Monocytes from controls and BD patients released lower levels of IL-1β (50% and 30%, respectively) under basal conditions, in comparison with exposure to LPS plus vehicle. In addition, increased levels of secreted IL-1β were observed in LPS-primed control monocytes treated with 5 µM thapsigargin comparatively to LPS plus vehicle. In contrast, LPS-primed monocytes from BD patients treated with tunicamycin (5 or 10 µg/mL) or thapsigargin (5 or 10 µM) released higher levels of IL-1β in comparison with LPS plus vehicle.

In brief, our results indicate that monocytes isolated from healthy controls and BD patients

release IL-1β in response to LPS. Furthermore, preliminary data suggest that a differential response to ER stress occurs between control and BD monocytes being BD patient-derived cells more susceptible than control. It is also possible that induction of ER stress may act as signal 2 for NLRP3 inflammasome activation after 8 h in BD patient-derived monocytes but not in control monocytes.

Table 5. Basal levels of IL-1β released by control and BD monocytes determined by ELISA kit (n=5, mean values ± SEM).

IL-1β (pg/mL)	
Healthy subjects	559.3 ± 228.6
BD patients	276.9 ± 75.4

Table 5 shows that basal levels of IL-1β were lower in monocytes isolated from BD patients than in monocytes from age- and gender-matched healthy control subjects. However, due to the great variability observed amongst the individuals enrolled in the study, IL-1β levels were not statistically different.

4.7.3. Expression of NLRP3 and pro-IL-1β in monocytes from healthy controls and BD patients

These preliminary findings (**Figure 33**) show that NLRP3 protein levels were about 4-fold higher in BD monocytes treated with 10 µM thapsigargin than under basal conditions. Moreover, NLRP3 levels also appear to be modulated by thapsigargin in control monocytes. However, it is important to note that due to the small size of the sample (n = 2), the differences were not statistically significant.

It was also observed a slight decrease in pro-IL-1β protein levels in tunicamycin-treated control and BD monocytes, in comparison with untreated cells. Interestingly, basal levels of pro-IL-1β determined in monocytes isolated from healthy control subjects were higher than those measured in BD monocytes, which can be related with the basal IL-1β levels secreted by

these cells. As previously mentioned (**Table 5**), under basal conditions, the levels of IL-1β were found higher in healthy control subjects and, by WB analysis, the protein levels of pro-IL-1β were also found higher in healthy control subjects. Thus, these preliminary results anticipate that healthy control subjects under basal conditions show higher protein levels of pro-IL-1β and, as consequence, higher levels of released IL-1β in relation to BD patients.
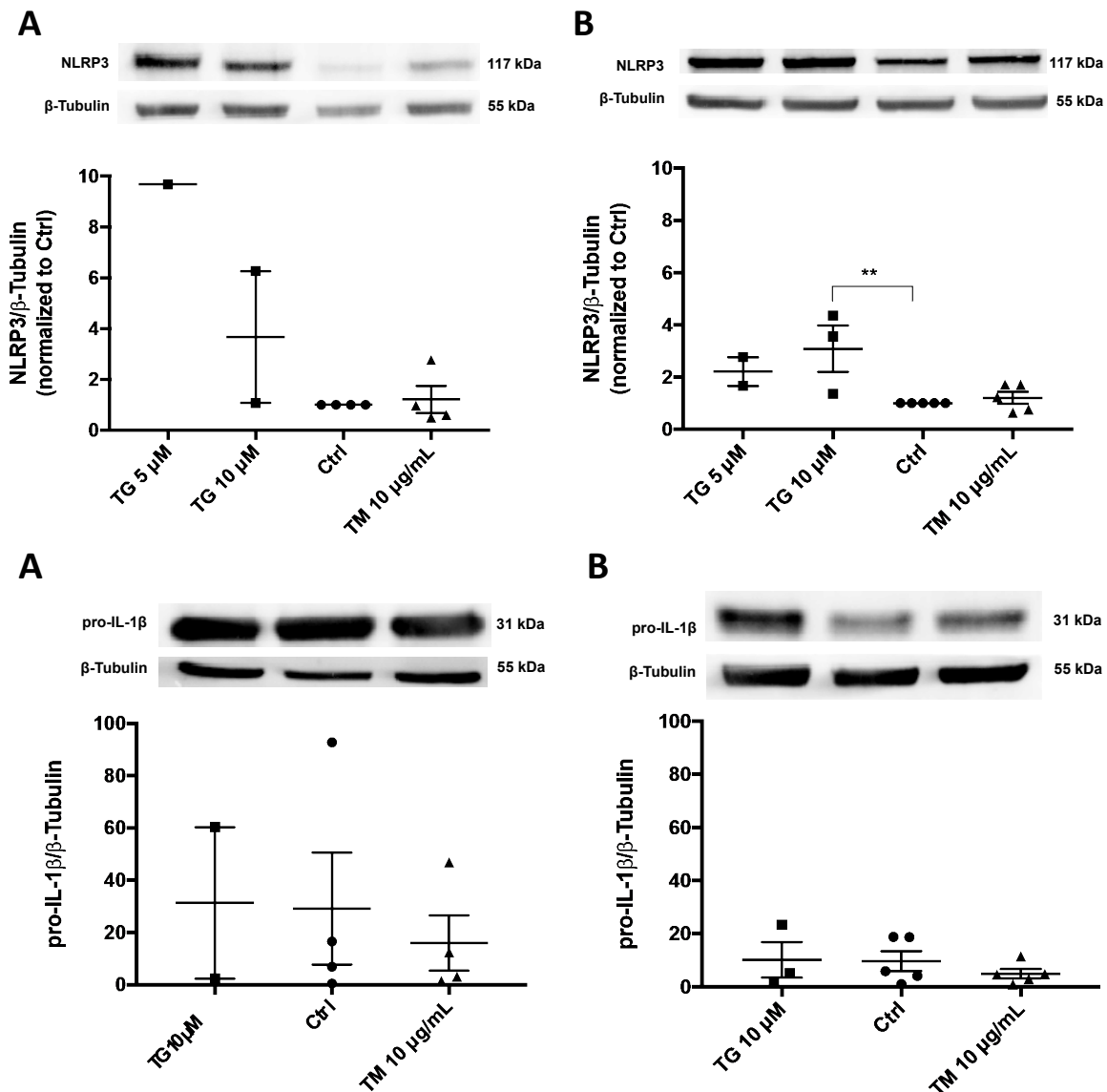
Figure 33. NLRP3 and pro-IL-1β protein levels under basal and ER stress-stimulated conditions in monocytes from healthy controls and BD patients. Protein levels of NLRP3 and pro-IL-1β were quantified by WB in total cellular extracts obtained from monocytes derived from healthy controls (A) and BD patients (B) treated with 10 µg/mL tunicamycin or thapsigargin (5 or 10 µM) for 8 h, in comparison with that determined under unstimulated conditions. β-Tubulin I was used as a protein loading control to normalize the levels of the protein of interest. NLRP3 protein levels were calculated relatively to control values. Results represent the means ± SEM of, at least, three independent experiments (with exception of 5 or 10 µM TG). Statistical significance between control (untreated cells) and treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test. ** p<0.01.

4.8. ER-mitochondria contacts during ER stress-induced NLRP3 activation in monocytes from healthy controls and BD patients

After obtaining evidences of the involvement of ER stress in the activation of the NLRP3 inflammasome in control and BD monocytes, which acts as priming and activation signals for its assembly, and given the relevance of MAMs on the innate immune responses (Missiroli et al, 2018), protein levels of two MAM-resident components (Sigma-1R and Mitofusin 2) were evaluated by WB (**Figure 34**) in monocytes obtained from BD patients and healthy controls to disclose the molecular mechanisms underlying ER stress-induced NLRP3 activation on these innate immune cells.
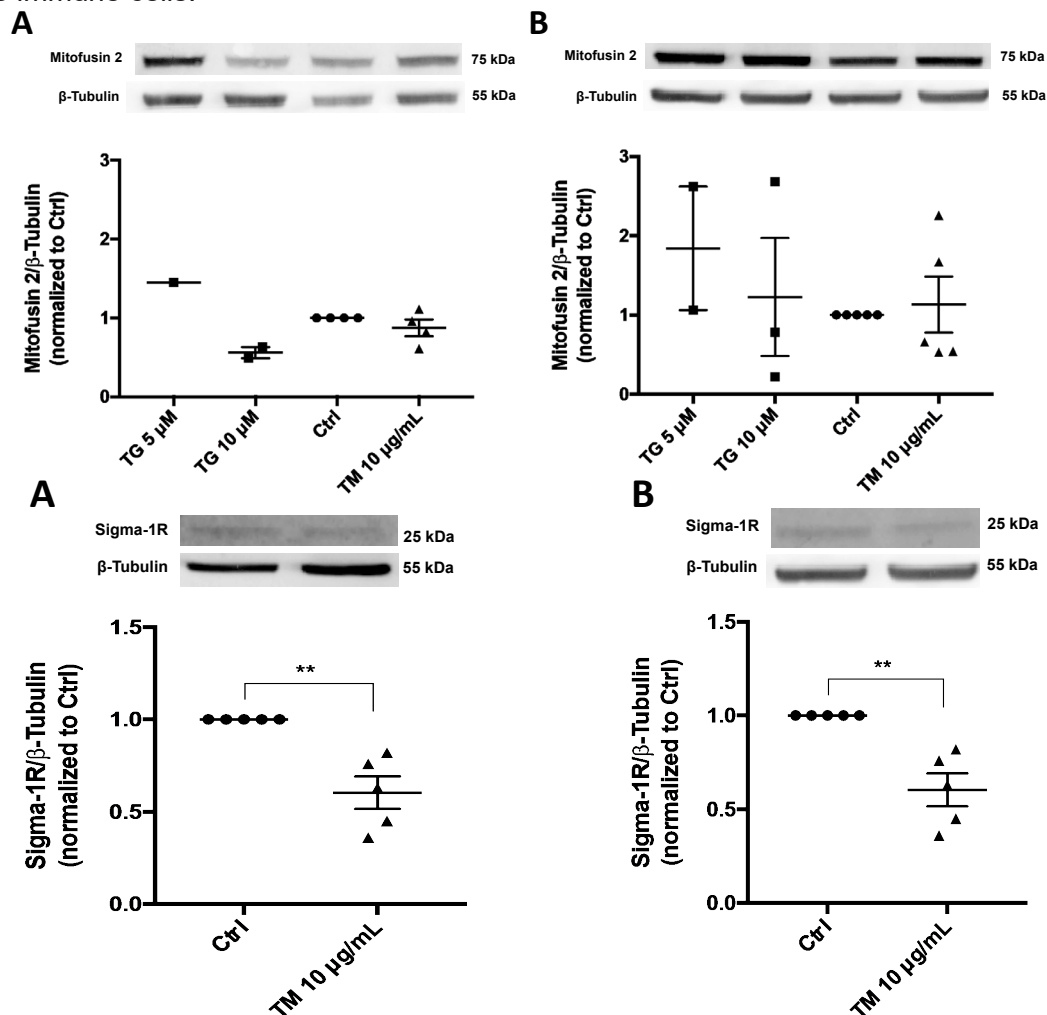
Figure 34. Sigma-1R and Mitofusin 2 protein levels in basal and ER stress conditions in monocytes from healthy controls and BD patients. Protein levels of Sigma-1R and Mitofusin 2 were quantified by WB in total cellular extracts obtained from monocytes derived from healthy controls (A) and BD patients (B) treated with 10 µg/mL tunicamycin or thapsigargin (5 or 10 µM) for 8 h and compared with that determined in control and BD monocytes under basal conditions. β-Tubulin I was used as a protein loading control to normalize the levels of the protein of interest. Results were calculated relatively to control values and represent the means ± SEM of at least three independent experiments (with exception of 5 or 10 µM TG). Statistical significance between control (untreated cells) and treated cells was determined using the one-way ANOVA test, followed by the Dunnett's post hoc test: **p<0.01.

Although without statistical significance, an increase in the protein levels of Mitofusin 2 was observed under tunicamycin- and thapsigargin-induced ER stress conditions in monocytes derived from BD patients in comparison with unstimulated cells. In the case of control healthy monocytes, a slight decrease in Mitofusin 2 protein levels was observed in treated conditions when compared with basal cells.

A decrease in Sigma-1R protein levels was found in control and BD monocytes under tunicamycin-induced ER stress. Importantly, a significant depletion of Sigma-1R was caused by tunicamycin treatment in BD patient-derived monocytes.

These preliminary results anticipate that ER-mitochondria contacts at MAMs are altered in BD patient-derived monocytes when exposed to stressful conditions.

Table 6. Basal levels of proteins involved in ER stress response, inflammasome activation and ER-mitochondria contacts detected by WB in control and BD monocytes (n=5, mean values ± SEM).

Protein	Healthy subjects	BD patients
ATF4	0.27 ± 0.10	0.39 ± 0.24
GRP78	1.72 ± 0.70	0.87 ± 0.21
NLRP3	16.26 ± 10.99	5.13 ± 2.60
pro-IL-1β	29.17 ± 21.46	9.70 ± 3.78
Mitofusin 2	3.15 ± 1.93	4.41 ± 3.71
Sigma-1R	0.55 ± 0.42	3.29 ± 3.17

Table 6 shows basal values of different proteins involved in the ER stress response, NLRP3 inflammasome activation and in ER-mitochondria contact analyzed in control and BD monocytes, which suggest that these pathways are affected in the innate immune system in BD but that also emphasize the need to increase the sample size in order to draw more robust conclusions.

4.9. *Clinical parameters of healthy control individuals and BD patients*

To disclose potential molecular mechanisms involved in BD pathophysiology, focusing the compromise of the innate immune system induced by ER stress, our research was complemented by several clinical analytical parameters (**Tables 7 and 8**) obtained in collaboration with CHUC, which may highlight some relevant information, namely, inflammatory parameters.

Table 7. Biochemical analysis and pharmacological therapy of healthy control individuals (n = 5) and BD patients (n = 5) under study. The values represented in bold with an asterisk (*) are higher than the correspondent reference values.

		Hemogram						Inflammatory parameters								
		Leukocytes (x109/L)	Neutrophils (x109/L)	Lymphocytes (x109/L)	Monocytes (x109/L)	Eosinophils (x109/L)	Basophils (x109/L)	C-reactive protein (mg/dL)	Procalcitonin (ng/mL)	Hemoglobin (g/dL)	Ferritin (ng/mL)	Creatine kinase (U/L)	Platelets (x109/L)	Mean platelet volume (fL)	Plateletcrit (%)	Drug Therapy
Healthy subjects	#1	5.7	3	1.9	0.4	0.4	0	0.03	0.01	14.7	22	----	190	10.3	0.20	------------
	#2	7.1	4.6	1.9	0.4	0.2	0	0.09	0.02	16.1	126	---	130	10.1	0.13	------------
	#3	6.1	4.2	1.1	0.6	0.1	0	0.2	0.04	15.1	167	58	243	8.8	0.21	------------
	#4	7.3	3.1	3.2	0.6	0.2	0.1	0.04	0.02	14.7	397*	---	230	8.4	0.19	------------
	#5	7.4	4.5	1.6	0.6	0.6	0.1	0.66*	0.08	15.1	145	---	192	8.7	0.17	------------
BD patients	#1	9.3	5.6	2.7	0.7	0.2	0.0	0.69*	0.03	15.3	169	128	324	324	0.22	Lamotrigine Clonazepam
	#2	5.9	4.0	1.3	0.5	0.0	0.0	0.09	0.02	15.2	69	100	287	287	0.22	Olanzapine
	#3	4.7	2.4	1.8	0.5	0.0	0.0	0.15	0.03	16.2	404*	310*	204	204	0.19	------------
	#4	12.5*	8.6*	2.8	0.8	0.2	0.1	0.14	0.01	14.7	184	300*	220	220	0.20	Valproic acid
	#5	7.8	4.0	2.8	0.9	0.1	0.0	0.02	0.01	15.7	252*	180*	208	208	0.19	Valproic acid

From the analysis of Table 7, it was observed that one of the DB patients exhibited high plasma levels of leukocytes, particularly neutrophils, which is indicative of leukocytosis. However, this increase was not detected in the other 4 patients and since the sample size was reduced, it cannot be inferred if this is a characteristic of BD patients. In addition, one subject from each group presented elevated levels of C-reactive protein and ferritin when compared to reference values, which may be a general marker of inflammation. High levels of ferritin in the absence of elevated hemoglobin, is probably associated with an inflammatory state. It was also observed that three of the five BD patients studied exhibited values of creatine kinase activity above the reference value, suggesting activation of creatine kinase enzyme in BD.

Table 8. Age and biochemical parameters analyzed on healthy individuals versus BD patients (n = 5, mean values ± SEM). Statistical significance between both groups was determined using the t-test.

Clinical parameters	BD patients	Control	p value
Age (years)	27.80 ± 2.20	28.20 ± 2.48	0.91
Leukocytes (x10^9/L)	8.04 ± 1.37	6.72 ± 0.34	0.38
Neutrophils (x10^9/L)	4.92 ± 1.05	3.88 ± 0.35	0.37
Lymphocytes (x10^9/L)	2.28 ± 0.31	1.94 ± 0.36	0.49
Monocytes (x10^9/L)	0.68 ± 0.08	0.52 ± 0.05	0.13
Eosinophils (x10^9/L)	0.10 ± 0.04	0.30 ± 0.09	0.08
Basophils (x10^9/L)	0.02 ± 0.02	0.04 ± 0.02	0.54
Protein C Reactive (mg/dL)	0.22 ± 0.12	0.20 ± 0.12	0.93
Procalcitonin (ng/mL)	0.02 ± 0.00	0.03 ± 0.01	0.32
Hemoglobin (g/dL)	15.14 ± 0.26	15.42 ± 0.25	0.46
Ferritin (ng/mL)	215.60 ± 55.44	171.40 ± 61.64	0.60
Creatine kinase (U/L)	203.60 ± 43.37	58	_
Platelets (x10^9/L)	248.60 ± 24.10	197 ± 19.71	0.14
Mean platelet volume (fL)	8.36 ± 0.51	9.26 ± 0.39	0.20
Plateletcrit (%)	0.20 ± 0.01	0.18 ± 0.01	0.18

Data from Table 8 show there weren't statistical differences between healthy control subjects and BD patients selected for this study, which belong to the same age group and have the same gender. Higher levels of ferritin were determined in BD patients in comparison with controls, in the absence of changes in hemoglobin values that were similar between the two groups. This result might be indicative of an inflammatory status, which is supported by the higher number of platelets determined in BD patients relatively to healthy individuals as well as an increase in immune cells such as monocytes.

Chapter 5
Discussion

Over the past years, the interest in understanding the molecular mechanisms underlying the pathophysiology of neuropsychiatric diseases, namely Bipolar disorder (BD), has significantly increased. As consequence, several studies have emerged reporting numerous alterations in BD patients, including impairment on innate immune responses. For instance, high prevalence of autoimmune diseases has been reported in BD patients and a different profile of immune cells as well as elevated levels of circulating immune markers such as pro-inflammatory cytokines have been described in individuals with BD diagnosis (Barbosa et al, 2014; Knijff et al, 2007; Modabbernia et al, 2013). Concomitantly, the last few years have witnessed a raise in the elucidation of the structure and function of MAMs, increasing the knowledge about the tethering proteins implicated in ER-mitochondria contacts and allowing to understand that the role of MAMs in mammalian cells is much more important than it would be anticipated, particularly as a mediator of cellular stress and inflammatory responses (Missiroli et al, 2018; van et al, 2014). Recent findings demonstrated that a MAMs-resident chaperone, Sigma-1R, plays an important role in neuropsychiatric diseases, which have also been associated with perturbed cellular resilience as consequence of mitochondria dysfunction and ER stress (Hashimoto, 2015; Mandelli et al, 2017). Taking the above-evidence into account, the present study was aimed to elucidate how the ER stress-MAMs-innate immunity axis is affected in BD investigating the impact of ER stress on innate immune cells, namely monocytes, with focus on the NLRP3 inflammasome, and the role of MAMs during ER stress-induced NLRP3 activation.

One of the main findings of this study is that ER stress induces NLRP3 inflammasome activation in human THP-1 monocytes. First, an ER stress model was established in THP-1 cells, a human monocyte cell line, and for that a dose- and time-dependent analysis of cellular viability and UPR induction was performed using pharmacological ER stressors, namely tunicamycin and thapsigargin. Experimental conditions able to trigger ER stress-induced UPR without evoking relevant cytotoxicity to human monocytes were selected (**Figures 15, 16 and 17**). Cellular viability was assessed by the resazurin assay, which represents an indirect measure of the number of viable cells since it is based on the evaluation of the metabolic activity (Ahmed et al, 1994; Prabst et al, 2017). Therefore, in the future, cell viability results should be complemented with the study of specific markers of cell damage, such as analysis of annexin

IV exposure by flow cytometry to detect early/late apoptotic and necrotic cell death. Accordingly, selected concentrations of tunicamycin and thapsigargin have been widely used by other groups as classical ER stress inducers able to activate the URP signaling pathways in THP-1 monocytes (Luis et al, 2014; Menu et al, 2012).

The first goal of this study was to unveil the role of the NLRP3 inflammasome on ER stress-induced innate immunity perturbations. We demonstrated that ER stress *per se* doesn't act as a signal 1 (priming step) to trigger the activation of the NLRP3 inflammasome in THP-1 monocytes since tunicamycin and thapsigargin were not able to release IL-1β. Accordingly, Tao and colleagues (Tao et al, 2018) showed that in the absence of LPS-priming, thapsigargin failed to promote the IL-1β release. The unchanged expression of NLRP3 inflammasome-related proteins that is required for its assembly, namely NLRP3 and pro-caspase-1, in THP-1 monocytes under ER stress conditions (**Figures 19 and 20**), further supports that ER stress *per se* does not act as a priming step (signal 1) for NLRP3 activation. Indeed, upregulation of NLRP3 inflammasome-related proteins is induced by a priming signal through the activation of inflammation-related transcription factors (Bauernfeind et al, 2009; Franchi et al, 2009; Huang et al, 2017), for instance NF-κB that is a well-known mediator of immune responses and its activation has been associated with ER stress induction. However, the mechanisms underlying ER stress-induced NF-κB activation remain controversial (Hung et al, 2004; Kim et al, 2013). For instance, Hu and colleagues demonstrated that ER stress activates NF-κB through the interaction between IRE1α and the IKK complex via the adaptor TRAF2 (Hu et al, 2006). On the other hand, Jiang and colleagues showed that the activation of NF-κB in response to ER stress is caused by phosphorylation of eIF2α (Jiang et al, 2003). To evaluate the involvement of NF-κB in ER stress-induced NLRP3 inflammasome activation in innate immune cells, the activity of this transcription factor was determined. Considering that only a modest increase in NF-κB activity was observed in tunicamycin- and thapsigargin-treated THP-1 cells (**Figures 22 and 23**) and that the NF-κB-dependent expression of NLRP3 inflammasome-related proteins was not affected under these conditions, it can be concluded that ER stress is not able to act as a first signal since it cannot trigger NF-κB activation in a significant and effective extent. Another possible hypothesis is that other inflammation-related transcription factors such as AP-1,

which mediates the expression of NLRP3 inflammasome-related proteins may be downregulated by ER stress in THP-1 cells (Hotamisligil, 2010). NF-κB activity was only assessed by WB analysis of phospho-IKBα and IKBα protein levels, since its activation is dependent on IKBα (Kretz-Remy et al, 1996; Miyamoto et al, 1994). To further explore how ER stress regulates the expression of inflammatory transcription factors, additional studies could be performed, such as analysis of NF-κB nuclear translocation using the standard confocal immunofluorescence microscopy and determination of AP-1 activity using gene reporter assays.

After tunicamycin treatment, protein levels of NLRP3 and pro-caspase-1 were identical to those detected in control conditions (**Figure 19**). However, treatment of THP-1 cells with thapsigargin under similar conditions decreased the levels of both NLRP3 and pro-caspase-1 approximately 50% relatively to untreated cells (**Figure 20**). Although NLRP3 expression is modulated by the NF-κB transcription factor (and by AP-1), its cellular levels are regulated by posttranscriptional modifications, namely ubiquitination and subsequent proteasome degradation (Bednash & Mallampalli, 2016; Py et al, 2013). Recently, Han and colleagues showed that NLRP3 is constitutively ubiquitinated by the subunit F-box L2 (FBXL2) of the Skp-Cullin-F box (SCF) family, which encodes RING domain-containing E3 ligases, to promote proteossomal degradation (Han et al, 2015). Accordingly, downregulation of NLRP3 protein levels induced by thapsigargin may be associated with increased proteasome activity or even an enhanced autophagy clearance to avoid NLRP3 inflammasome activation. This increase is supported by findings reporting an enhance of ERAD and proteasome activity as well as autophagy induction in response to mild-moderate ER stress, to promote clearance of unfolded/misfolded proteins from the ER lumen and, therefore, restore proteostasis and preserve cellular homeostasis (Lee et al, 2015; Ogata et al, 2006). In response to harmful stimuli, such as chronic or severe ER stress, the NLRP3 protein is deubiquitinated, which triggers inflammasome assembly and activation (Bednash & Mallampalli, 2016; Py et al, 2013). In the future, it would be interesting to investigate NLRP3 ubiquitination in THP-1 monocytes under ER stress conditions by WB and to correlate NLRP3 levels and inflammasome activation with proteasome activity, which can be achieved using proper substrates of the proteasome and/or pharmacological approaches, for instance the MG 132 proteasome inhibitor. By using an autophagy inhibitor such as 3-meth-

yladenine, it will be possible to explore the hypothesis that NLRP3 is degraded in the lysosome in thapsigargin-treated THP-1 cells.

Upon excluding ER stress as a first signal for NLRP3 inflammasome activation in THP-1 monocytes, NLRP3 activity was further assessed after LPS-priming for activation of inflammatory transcription factors and upregulation of inflammasome components (Guo et al, 2015; Hanamsagar et al, 2012; He et al, 2016; Walsh et al, 2014). Indeed, our data demonstrate that ER stress acts as a second signal (activation step) able to promote NLRP3 inflammasome assembly and activation in THP-1 monocytes since both tunicamycin and thapsigargin induced pro-IL-1β cleavage resulting in the release of IL-1β in LPS-primed cells (**Figure 18**). By measuring secreted IL-1β levels by WB analysis, Menu and colleagues reported that tunicamycin induced NLRP3 inflammasome activation in THP-1 monocytes and in human primary monocytes whereas thapsigargin exclusively activated the NLPR3 inflammasome in primary human monocytes (Menu et al, 2012). The reason for this discrepancy may be related with different methodologies used for quantification of IL-1β levels in supernatants. In fact, ELISA assay is much more sensitive than WB analysis for IL-1β quantification.

After establishing a relationship between ER stress induction and innate immunity perturbations in THP-1 monocytes, which was shown to occur in a NLRP3 inflammasome-dependent manner, MAM's dysfunction was evaluated as a potential link between ER stress and NLRP3 inflammasome activation. The analysis of MAM's structure by WB revealed that ER-mitochondria contacts are altered upon ER stress in THP-1 monocytes. However, MAM's proteins were found to be differentially regulated by tunicamycin and thapsigargin in these cells. Tunicamycin slightly upregulated the MAMs-resident chaperone Sigma-1R in the absence of changes in the levels of the ER-mitochondria tethering protein Mitofusin 2 (**Figure 24**). On the other hand, thapsigargin-induced ER stress strongly upregulated Mitofusin 2 while Sigma-1R levels were unaffected (**Figure 25**). Recently, it was demonstrated that ER stress rapidly upregulates the MAMs-resident chaperone Sigma-1R, which is associated with repression of ER stress-induced cell death signals (Hayashi & Su, 2007). In dormant state, Sigma-1R binds to the GRP78 molecular chaperone on the ER membrane. Upon ER stress, Sigma-1R dissociates from GRP78 and interacts with other ligands, modulating the activity of the ER stress

sensors PERK, IRE1α, and ATF6. Furthermore, under ER stress conditions, Sigma-1R can interact with IP3R, stabilizing their structure and modulating ER-to-mitochondria Ca^{2+} transfer (Hashimoto, 2015; Nguyen et al, 2015). Accordingly, a growing number of functions have been described for this chaperone specifically enriched at MAMs, including regulation of ion channels such as Ca^{2+} and K^+ channels, modulation of neurotransmitters release and of kinases activity and regulation of lipids distribution (Hashimoto, 2015; Nguyen et al, 2015; Omi et al, 2014). Hayashi and Su have shown recently that both tunicamycin and thapsigargin ER stressors upregulate Sigma-1R. In addition, these authors demonstrated that the long Sigma-1R half-life as well as its stability and subcellular distribution are altered upon ER stress, showing that a brief exposure to stress conditions upregulated Sigma-1R for more than 24 h whereas longer ER stress exposure (more than 3 h) was not able to upregulate Sigma-1R, suggesting that the stability of Sigma-1R protein and/or mRNA declined under chronic ER stress (Hayashi & Su, 2007). Accordingly, it can be hypothesized that the absence of a significant increase of Sigma-1R levels in response to ER stress in THP-1 cells results from chronic exposure to tunicamycin and thapsigargin. Indeed, a subtle increase in Sigma-1R levels was detected in THP-1 monocytes treated with these ER stress inducers for 1 h. Upregulation of Mitofusin 2 in thapsigargin-treated monocytes may be explained based on its modulatory effect on intracellular Ca^{2+} homeostasis. Thapsigargin promotes ER stress through blockage of the ER Ca^{2+}-ATPase (SERCA), leading to the depletion of ER Ca^{2+} stores (Deniaud et al, 2008; Zhao et al, 1996). As consequence of ER Ca^{2+} release, cytoplasmic Ca^{2+} concentration increases. Ca^{2+} is a second messenger that plays pivotal roles within cells since it regulates multiple signaling pathways. However, cytosolic Ca^{2+} concentration needs to be tightly regulated since a high Ca^{2+} rise can trigger catastrophic events that committee cells to death (Gurung et al, 2015; Krebs et al, 2015). Mitofusin 2 is transmembrane GTPase that functions as a bridge to promote ER-mitochondria tethering due to its location in both the outer mitochondrial membrane and the ER membrane (Ainbinder et al, 2015). ER-mitochondria contacts are crucial to preserve cell survival under stress conditions since they allow Ca^{2+} transfer from the ER to mitochondria to maintain mitochondrial metabolism (Kamishima & Quayle, 2002). Excessive ER-to-mitochondria Ca^{2+} transfer under chronic stress conditions can lead to mitochondrial Ca^{2+} overload

activating the mitochondrial apoptotic cell death pathway (Fribley et al, 2009). Therefore, Mito-fusin 2 upregulation induced by thapsigargin in THP-1 cells can occur as an adaptive response upon ER Ca^{2+} depletion to increase ER-mitochondria contacts and promote Ca^{2+} transfer thus avoiding the rise of cytosolic Ca^{2+}, and simultaneously preserve ATP production required to deal with stressful conditions. This hypothesis is supported by findings showing an increase of mitochondrial uptake as consequence of high Ca^{2+} cytoplasmic levels and by evidences demonstrating the high efficiency of mitochondrial Ca^{2+} uptake mediated by Mitofusin 2, which overcomes the low affinity Ca^{2+} uptake mechanism mediated by the mitochondrial Ca^{2+} uni-porter (MCU) (Ainbinder et al, 2015; Kamishima & Quayle, 2002). Interestingly, Ainbinder and colleagues demonstrated that Mitofusin 2 knockdown results in mitochondrial depolarization suggesting that it can preserve the mitochondrial membrane potential (Ainbinder et al, 2015). In thapsigargin-treated THP-1 cells, Mitofusin 2 up-regulation (**Figure 25**) occurred simulta-neously with the preservation of mitochondrial membrane potential, as evaluated with the flu-orescent probe TMRE (**Figure 28**); therefore, it can be hypothesized that monocytes treated with thapsigargin don't exhibit a depolarization of mitochondrial membrane due to presence of increased Mitofusin 2 levels. Concomitantly, it can also be suggested that tunicamycin-induced depolarization of the mitochondrial membrane (**Figure 28**) occurs because Mitofusin 2 is not upregulated upon exposure to this ER stressor (**Figure 24**).

To unveil the role of MAMs on ER stress-induced NLRP3 activation in human THP-1 mono-cytes, mitochondrial ROS production was evaluated (**Figure 27**). Only thapsigargin promoted a subtle, not statistically significant, increase in the levels of mitochondrial ROS, which were measured with the fluorescent probe MitoSOX. Using the peroxide-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (H2DCFDA) instead of MitoSOX, Chen and colleagues have shown that exposure to thapsigargin-induced ER stress didn't signifi-cantly increase ROS levels in THP-1 macrophages (Chen et al, 2017). On the other hand, an increase in ROS production in response to ER stress has been reported using H2DCFDA in other cell models (Dolai et al, 2011; Toral et al, 2017). While MitoSOX detects mitochondrial superoxide production, the H2DCFDA probe quantifies the levels of intracellular ROS, partic-ularly hydrogen peroxide (Wojtala et al, 2014). A slight decrease in mitochondrial ROS levels

was observed upon tunicamycin treatment (**Figure 27**). Tunicamycin-induced ER stress has been associated with elevated expression of antioxidant enzymes, such as the mitochondrial superoxide dismutase 2 (SOD2) that plays a protective role against oxidative stress (Srivastava et al, 2010) and mitochondrial uncoupling proteins (UCPs), namely UCP-3, which are located in the inner mitochondrial membrane and modulate mitochondrial ROS production (Cardoso et al, 2015). Therefore, it can be suggested that upregulation of the mitochondrial antioxidant enzyme SOD2 and/or UCP3 can explain the decreased levels of ROS observed in tunicamycin-treated cells. However, it cannot be ruled out that other antioxidant systems or UCPs might be involved. Further studies are necessary to prove this hypothesis.

Considering that MAMs are lipid raft-like subdomains involved in lipid metabolism, particularly phospholipid synthesis, the modulatory role of ER stress on the lipid profile of innate immune cells was evaluated (Area-Gomez et al, 2012; Missiroli et al, 2018). As expected, different relative abundances were observed among the various classes of phospholipids in THP-1 cells (Martins et al, 2016). In addition, the preliminary data suggests that ER stress modulates the relative abundance of several phospholipids, namely cardiolipin (**Figure 26**). Given that mitochondrial dysfunction has been consistently associated with NLRP3 inflammasome activation, and cardiolipin is a phospholipid exclusively found in the inner mitochondrial membrane of eukaryotic cells, a putative relationship between NLRP3 inflammasome activation and cardiolipin has been highlighted in the last few years. Cardiolipin has been recently described as a NLRP3 inflammasome activating stimulus through its direct binding to NLPR3 (Gong et al, 2018; Iyer et al, 2013; O'Neill, 2013). Additionally, ROS production has been proposed as an event occurring upstream of cardiolipin release by mitochondria during NLPR3 inflammasome activation since both ROS-dependent and -independent NLRP3 activation require mitochondrial cardiolipin release. Moreover, Iyer and colleagues described two different roles played by cardiolipin in NLRP3 inflammasome activation: it can function as a platform for docking NLRP3 to the mitochondria, but it can also directly activate the NLRP3 inflammasome (Iyer et al, 2013). In the presence of danger stimuli, cardiolipin that is normally localized in the inner mitochondrial membrane, translocates to the outer mitochondrial membrane leading to NLRP3 inflammasome activation. Therefore, when mitochondria are dysfunctional, externalization of

cardiolipin acts as a danger signal. Furthermore, the translocation of cardiolipin to the outer mitochondrial membrane also promotes mitochondrial fission (Gong et al, 2018; Iyer et al, 2013; O'Neill, 2013). Although a decrease in global cardiolipin levels was observed during ER stress-induced NLRP3 inflammasome activation in THP-1 monocytes, it would be important to monitor mitochondrial levels of this lipid under these conditions as well as its externalization. In fact, the decrease in the number of mitochondria, as supported by enhanced mitophagy detected by electron microscopy, could explain the depletion of cardiolipin observed in treated cells.

Qualitative ultrastructure analysis with a special focus on ER and mitochondria morphology was performed and also supports the deleterious effect of ER stress induction in human monocytes (**Figure 29**). Upon ER stress, THP-1 monocytes exhibited various morphological alterations, including decreased cellular projections, presence of dilated ER and tubular mitochondria, enhanced mitochondria fission and autophagy-mediated clearance (mitophagy) events and also an increase of vacuoles and autophagic structures. Oslowski and Urano, among others, have already observed by electron microscopy that ER stress is associated with a remarkably enlargement of the ER in cells and tissues (Montalbano et al, 2013; Oslowski & Urano, 2011). The significant increase of autophagic structures detected in tunicamycin- and thapsigargin-treated monocytes are in accordance with Ogata and colleagues' studies that found an accelerated autophagosome formation in response to ER stress (Ogata et al, 2006). Together, these results suggest that autophagy is activated when cells are submitted to ER stress to clear misfolded protein and dysfunctional organelles with the purpose of preserving cell survival (Kincaid & Cooper, 2007). Vannuvel and colleagues also demonstrated that ER stress affects the mitochondrial morphology, increasing fission processes and mitophagy. It is believed that ER stress induces mitochondrial fission to remove damaged mitochondria through mitophagy (Vannuvel et al, 2013). In the future, it would be interesting to evaluate ER-mitochondria contacts by electron microscopy, but with a higher resolution, or by other techniques such as immunogold labeling. In addition, the qualitative ultrastructure study in THP-1 cells under ER stress conditions could have been complemented by WB analysis of fission (e.g. Drp1) and mitophagy (e.g. Parkin) markers.

After investigating the role of ER stress in innate immunity using human THP-1 mono-cytes, focusing on the NLRP3 inflammasome activation, we proposed to disclose how the ER stress-NLRP3 inflammasome axis is modulated in BD patients-derived monocytes. Further-more, MAM's dysfunction was also analyzed as the putative link between ER stress and in-flammasome activation in this patient-derived cellular model. It is noteworthy that it represents a pioneer exploratory study aimed to clarify the molecular mechanisms underlying BD patho-physiology. The preliminary results are promising but should be explored using an enlarged number of patients and matched controls.

First, ER stress was induced in monocytes isolated from healthy controls and BD patients, taking into consideration the results obtained in the THP-1 cell line of human monocytes. A dose- and time-dependent effect of the pharmacological ER stress inducers on UPR markers and cell viability should have been performed, however, the reduced number of monocytes obtained from approximately 20 ml of peripheral blood collected from controls and BD patients limited the experimental conditions that were tested. Therefore, the experimental conditions used on this set of experiments were the same previously selected for THP-1 cells. Levels of markers of ER stress-induced UPR were evaluated in control and BD monocytes treated with tunicamycin and thapsigargin for 8 h and the differences achieved between these two groups were without statistical significance (**Figure 30**). Pfaffenseller and colleagues found an altered ER stress response in monocytes derived from late-stage BD patients while monocytes from early-stage patients didn't differ from healthy subjects (Pfaffenseller et al, 2014). The present findings are in accordance with Pfaffenseller and colleagues since patients enrolled in this study were diagnosed with early BD. The absence of GPR78 upregulation 8 h after ER stress induction can be explained by the fact that the expression of this chaperone is a late response to the activation of the three UPR pathways (Baumeister et al, 2005). Indeed, in tunicamycin- and thapsigargin-treated THP-1 monocytes, GRP78 levels increased after ATF4 upregulation, which occurs downstream of the PERK-mediated UPR branch activation (**Figures 16 and 17**).

One of the main findings of this exploratory study is that, contrary to what was observed in THP-1 monocytes, ER stress *per se* acts as signal 1 and 2 for NLRP3 inflammasome ac-tivation after 32 h, but not upon 8 h, in primary monocytes isolated from healthy controls and

BD patients (**Figure 31**). The discrepancy in the immune response between human THP-1 monocytes and primary monocytes may be related to the fact that the later cells have intrinsic danger signals due to the presence of invasive pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), which increase the levels of pro-inflammatory transcription factors such as NF-κB that in turn up-regulate NLRP3 and pro-IL1β enhancing the susceptibility to subsequent stimuli that promote inflammasome assembly. This hypothesis is supported by other studies showing that human primary immune cells are more responsive to ER stress than the THP-1 cell line (Menu et al, 2012) and by our results demonstrating that LPS priming, a PAMP present in several bacteria, activates the NLRP3 inflammasome in THP-1 cells. Release of IL-1β was observed in LPS-primed control- and BD patient-derived monocytes in comparison with unstimulated cells (**Figure 32**). Accordingly, BD patients-derived monocytes seem more responsive to ER stress conditions than control monocytes subjected to the same experimental conditions, however, it is imperative to increase the number of subjects to draw robust conclusions. These results are supported by several findings implicating dysfunction of the innate immune system in BD pathophysiology. Indeed, high prevalence of autoimmune diseases such as Crohn's disease and autoimmune hepatitis has been described in BD patients, which seem to exhibit an immune cell profile distinct from that of healthy controls. In addition, BD patients have also been shown to have elevated levels of circulating inflammation markers including IL-4 and IL-10 (anti-inflammatory cytokines) and IL-1β and IL-6 (pro-inflammatory cytokines) and an unbalance between IL-1β and IL-6 production (Barbosa et al, 2014; Knijff et al, 2007; Modabbernia et al, 2013).

Interestingly, the results suggest that ER stress modulates the levels of NLRP3 inflammasome-related proteins in monocytes from healthy controls and BD patients (**Figure 33**). Moreover, it was also observed that BD patient-derived monocytes have lower basal levels of IL-1β and pro-IL-1β in comparison with control cells (**Table 5 and Figure 33**). According to the literature, there is no consistent evidence concerning IL-1β and pro-IL-1β levels in BD. Barbosa and colleagues didn't find significant differences in IL-1β content between BD patients and control subjects (Barbosa et al, 2014). On the other hand, Modabbernia and colleagues reported elevated circulating levels of pro-IL-1β and IL-1β in BD patients comparatively to

controls (Modabbernia et al, 2013). Although Goldsmith et al didn't observe significant differ-ences in IL-1β levels between BD patients in the depression state and controls, they reported a significant increase of IL-1β secretion in euthymic BD patients (Goldsmith et al, 2016). Based on the above evidence, this study revealed relevant information that, while preliminary and in need of further investigation, contributes to explore the molecular mechanisms underlying BD pathophysiology, especially ER stress-mediated immune alterations.

ER-Mitochondria communication at MAMs during ER stress-induced NLRP3 activation was also assessed in BD patients versus healthy controls. Our preliminary results anticipate that ER-mitochondria contacts at MAMs are altered in BD patients exposed to ER stress, since an increase in Mitofusin 2 and a decrease in Sigma-1R protein levels was observed in relation to matched controls (**Figure 34**). As previously mentioned, Mitofusin 2 upregulation can occur to increase the ER-mitochondria contacts promoting the mitochondrial Ca^{2+} uptake from ER, to avoid Ca^{2+} imbalance upon ER Ca^{2+} release to the cytosol and to maintain mitochondrial met-abolic activity (Ainbinder et al, 2015; Kamishima & Quayle, 2002). However, Ca^{2+} overload can lead to mitochondrial dysfunction, which can release DAMPs such as ROS, mtDNA and cardi-olipin to trigger NLRP3 inflammasome activation (Gurung et al, 2015). Based on exposed, it is of utmost importance that Ca^{2+} transfer between ER and mitochondria will be investigated and its impact on NLRP3 activation will be evaluated in both human THP-1 and primary monocytes. For this purpose, ER and mitochondrial Ca^{2+} content could be determined with specific fluo-rescent probes. Furthermore, the NLRP3 inflammasome activation should be measured in the presence of specific inhibitors of ER Ca^{2+} release (e.g. dantrolene and xestospongin C) and of the MCU (e.g. RU360), which mediates mitochondrial Ca^{2+} uptake. Besides that, both NLRP3 activation and modulation of ER-to-mitochondria Ca^{2+} transfer should also be assessed when Mfn2 is silenced. In addition, the release of other mitochondrial damage-associated molecules such as cardiolipin should also be evaluated in control and BD monocytes under ER stress conditions. The determination of levels and sub-cellular localization of calreticulin, a protein localized predominantly in the ER that plays a key role in intracellular Ca^{2+} homeostasis, would also be relevant in the context of the present study. Recently, the surface-exposed calreticulin has been pointed as a DAMP to activate the immune system (Zhang et al, 2014). Our prelimi-

nary data suggesting that BD patients have decreased Sigma-1R levels support the described role of Sigma-1R in the etiology and treatment of neuropsychiatric disorders. Ample evidence, including the presence of genetic variants within the SIGMAR1 gene in patients and the interaction of numerous antidepressants with these receptors, further suggest a role of Sigma-1R in affective disorders (Hashimoto, 2015; Mandelli et al, 2017). Given that the MAM-resident Sigma-1R chaperones can regulate several cellular processes including the UPR activation in order to protect cells from ER-mediated cellular stress, and considering that the Sigma-1R activation by agonists could prevent proteins misfolding (Tsai et al, 2014), the induction of Sigma-1R activity using pharmacological approaches could prevent the deleterious effects of chronic ER stress. Therefore, it would be interesting to evaluate NLRP3 inflammasome activation in both tunicamycin- and thapsigargin-treated THP-1 monocytes and primary BD patients-derived monocytes in the presence of Sigma-1R agonists.

Finally, to achieve a better understanding of BD pathophysiology, the *in vitro* studies were complemented by several analytical parameters determined in the plasma of patients and matched controls, with a focus on inflammatory parameters (**Tables 7 and 8**). It was found that BD patients have an increased activity of creatine kinase, however, this increase could be induced by antiepileptic drugs or even by micro-injuries induced by exercise (Jiang et al, 2016). Moreover, it was noticed that although hemoglobin levels are not affected in BD patients, increased values of ferritin were detected, suggesting that this increase is not due to anemia (Jung et al, 2017) and therefore can be associated with an inflammatory status. This association is supported by evidence showing that ferritin levels can be significantly increased in response to inflammation as consequence of cell stress and damage (Kell & Pretorius, 2014) and by our findings reporting an increase in immune cells such as monocytes. Moreover, higher number of platelets was detected in BD patients relatively to healthy individuals (**Table 8**). Mert and Terzi also reported that inflammatory cells, such as platelet count, are altered during mania episodes, which supports the hypothesis that inflammatory activation occurs in BD pathology (Mert & Terzi, 2016). Interestingly, it should be noted that classical mood stabilizers, such as valproic acid, have been shown to exhibit anti-inflammatory properties, modulating the systemic and CNS inflammatory responses (Muneer, 2016). All over again, it is important

to emphasize that this is an exploratory study and additional experiments in an enlarged sample of healthy and BD patients are required to further support a relationship between BD and inflammation.

Chapter 6
Conclusions and Future perspectives

Several events including impaired cellular resilience, compromised ER stress response, mitochondrial dysfunction and innate immune disturbances have been implied in the initiation and/or progression of BD pathophysiology. Based on these assumptions, this master thesis was focused on deciphering whether the NLRP3 inflammasome deregulation is a consequence of ER stress in BD and in investigating the putative MAM's dysfunction as the link between ER stress and NLRP3 inflammasome activation in BD. In a first approach the THP-1 monocytic cell line was used as a preliminary *in vitro* model to deeply explore the molecular mechanisms evoked during ER stress in the innate immune system. Importantly, the more relevant findings achieved in the human cell line were also disclosed in BD patients-derived monocytes. For this purpose, cells were treated with two well-established ER stressors, tunicamycin and thapsigargin (ER stress conditions), and were compared with unstimulated cells (basal conditions).

Overall, the major findings achieved in the THP-1 monocytic cell line include:

o   Tunicamycin and thapsigargin are suitable pharmacological approaches to induce an *in vitro* ER stress model in innate immune cells, particularly after 8 h of treatment, as demonstrated by the activation of the UPR signaling pathways PERK and IRE1α.

o   ER stress induction *per se* is not a signal 1 (priming step) for NLRP3 inflammasome activation and subsequent IL-1β release, probably because the modulation of the transcription factor NF-κB does not efficiently translate into up-regulation of NLRP3 inflammasome related proteins, namely NLRP3 and pro-caspase-1.

o   After pulsing cells with the signal 1 LPS, ER stress acts as a signal 2 (activation step) for NLRP3 inflammasome assembly, which was confirmed by enhanced pro-IL-1β processing and IL-1β release.

o   ER-mitochondria contacts at MAMs are altered upon ER stress, however, tunicamycin and thapsigargin differently regulate MAM's composition. Indeed, tunicamycin slightly increases Sigma-1R levels without affecting Mitofusin 2 levels, and thapsigargin up-regulates Mitofusin 2 and doesn't modulate Sigma-1R protein levels.

o   ER stress modulates MAMs functioning. Of relevance, tunicamycin-induced depolar-

ization of the mitochondrial membrane suggest that alterations of mitochondrial membrane potential may be behind tunicamycin-induced NLRP3 inflammasome activation.

o ER stress seems to modulate monocyte's lipid profile, namely of cardiolipins that are uniquely found in mitochondria. Externalization of cardiolipin to the outer mitochondrial membrane upon mitochondrial damage can be involved in ER stress-induced NLRP3 inflammasome activation.

o ER stress induces several morphological alterations in immune cells, including decreased cellular projections, dilated ER and a significant increase of vacuoles and autophagic structures, suggesting the induction of autophagy. Mitochondrial dynamics is also affected by ER stress, which was confirmed by a significant increase on tubular mitochondria, mitochondria fission and mitophagy.

Overall, the major findings in control- and BD patient-derived monocytes include:

o Tunicamycin and thapsigargin are suitable tools to trigger ER stress in monocytes derived from BD patients and healthy controls, mainly after 8h of treatment, confirmed by the activation of the PERK UPR signaling pathway.

o In contrast to the results obtained in THP-1 cells, ER stress *per se* induces the NLRP3 inflammasome activation in control and BD monocytes for long time periods (32 h), indicating that ER stress acts as signal 1 and 2 for NLRP3 inflammasome activation. By opposition, ER stress does not work as signal 1 for NLRP3 inflammasome activation in short time periods (8 h).

o LPS-primed BD patient-derived monocytes seem more responsive to ER stress than control monocytes.

o ER-mitochondria contacts at MAMs are altered in BD monocytes subjected to ER stress conditions. Indeed, BD patients exhibited increased Mitofusin 2 protein levels and downregulation of Sigma-1R, relatively to control monocytes.

o The analytical parameters analyzed suggest that BD patients are more susceptible to a pro-inflammatory status than healthy individuals.

Taking in account the above evidences, it can be concluded that ER stress activates the NLRP3 inflammasome in human THP-1 monocytes as well as in monocytes derived from BD patients and healthy controls. Additionally, our results suggest that MAM's composition is affected in THP-1 and BD patient-derived monocytes upon ER stress. However, sample size of controls and BD patients should be enlarged to validate these preliminary results.

In the future, it will be of relevance to confirm and validate the association between ER stress and NLRP3 inflammasome activation in BD patients, as well as to disclose the putative role of MAMs as the link between ER stress and inflammasome activation in BD. For that, functional and structural parameters mediated by MAMs should also be analyzed in monocytes derived from controls and BD patients, namely mitochondrial ROS production, mitochondrial membrane potential and mitochondrial cardiolipin content.

Furthermore, in order to obtain a direct correlation between ER stress-induced NLRP3 inflammasome activation and the molecular mechanisms underlying its activation in THP-1 monocytes and human BD monocytes, it would be of interest: (1) to clarify the role of the transcription factor NF-κB in NLRP3 inflammasome activation upon ER stress, using pharmacological or molecular approaches able to inhibit the NF-κB and/or to track the NF-κB nuclear translocation; (2) to assess the role of mitochondrial ROS production in ER stress-induced NLRP3 inflammasome activation, for instance after treating the cells with mitochondrial antioxidants; (3) to disclose the role of MAMs in ER stress-induced NLRP3 inflammasome activation, by potentiating or inhibiting the expression of MAM's components, using pharmacological and molecular approaches; (4) to evaluate other molecular signals functioning as immune system DAMPs, as potential activators of NLRP3 inflammasome with a special focus on calreticulin, cardiolipin externalization, mtDNA release and k^+ efflux; (5) to determine the NLRP3 inflammasome activation in the presence of specific inhibitors of ER Ca^{2+} release and of mitochondrial Ca^{2+} uptake; (6) to complement the qualitative ultrastructure analysis in THP-1 cells under ER stress conditions with WB analysis of fission and mitophagy markers and/or immunogold labeling to measure ER-mitochondria contacts; (7) to correlate NLRP3 levels with its ubiquitination levels and with proteasome activity; (8) to correlate the *in vitro* findings with the clinical features of BD patients as well as with therapeutics.

In conclusion, this research work may contribute to elucidate the molecular mechanisms underlying BD pathophysiology, which ultimately could shed light for novel therapeutic strategies and new diagnostic biomarkers.

Chapter 7
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