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Elucidating the Mechanisms Underlying How VMP1 Regulates Inflammatory Responses

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ELUCIDATING THE MECHANISMS UNDERLYING HOW VMP1 REGULATES

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BY

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To my Grandma Rose who has been everything to me.

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LIST OF ABBREVIATIONS

4-parameter logistic	4PL
α-synuclein	α-syn
Adenosine 5-triphosphate disodium salt hydrate	ATP
Alzheimer's disease	AD
American type culture collection	ATCC
Antigen presenting cells	APCs
Apoptosis-associated speck-like protein containing a CARD	ASC
Autophagic secretion of mitochondria	ASM
Autophagosome-lysosome pathway	ALP
Beclin-1	BECN1
Blood brain barrier	BBB
Bone marrow-derived macrophages	BMDMs
Bovine serum albumin	BSA
Calcium/calmodulin-dependent protein kinase II	CaMKII
Calcium induced calcium release	CICR
Calmodulin	CaM
Cell-free mitochondrial DNA	cfmtDNA
Central nervous system	CNS
Cerebrospinal fluid	CSF

Cyclic GMP-AMP synthase	cGAS
Cyclic guanosine monophosphate-adenosine monophosphate	cGAMP
Damage associated molecular patterns	DAMPs
Dopaminergic	DAergic
Double-stranded RNA	dsRNA
Dynamin-related protein 1	DRP1
Endoplasmic reticulum	ER
EV multiplex analysis of colocalization	EV-MAC
Extracellular vesicles	EVs
Fetal bovine serum	FBS
Fluorochrome-labeled inhibitors of caspases assay	FLICA
G-protein coupled receptors	GPCR
Galectin-3	Gal-3
Gasdermin D	GSDMD
Gasdermin E	GSDME
Geneticin	G418
H^+-Ca^{2+} exchangers	HCX
High mobility group box 1	HMGB1
Horseradish peroxidase	HRP
IκB kinase	IKK
Inner mitochondrial membrane	IMM
Inositol 1,4,5-trisphosphate receptor channels	IP3Rs

Interferon gamma	IFN-γ
Interleukin 1β	IL-1β
Interleukin 18	IL-18
Interleukin 6	IL-6
Isolation membranes	IMs
Knock out	KO
Lactate dehydrogenase	LDH
Leucine-rich repeat	LRR
Lipopolysaccharide	LPS
Lysosome-associated membrane protein 1	LAMP1
Major Histocompatibility Complex	MHC
Mammalian target of rapamycin	mTOR
Membrane contact sites	MCSs
Microtubule-associated protein 1A/1B-light chain 3	LC3
Mitochondria-associated membrane	MAM
Mitochondrial Ca^{2+} Uniporter	MCU
Mitochondrial DNA	mtDNA
Mitochondrial NCX	mNCX
Mitochondrial permeability transition pore	mPTP
Mitochondrial reactive oxygen species	mtROS
Mitofusin 1	MFN1
Mitofusin 2	MFN2

Mitogen-activated protein kinase	MAPK
MitoTracker Green FM	MTG
MitoTracker Red CMXRos	CMXRos
Multiple sclerosis	MS
Myeloid differentiation primary response protein 88	MyD88
Na^+-Ca^{2+} exchanger	NCX
NOD-like receptor	NLR
Nucleic acid-sensing	NA-sensing
Nucleotide-binding domain	NACHT
Nucleotide-binding domain, leucine-rich repeats containing family, pyrin domain-containing-3	NLRP3
Optic atrophy protein 1	OPA1
Oxidative phosphorylation	OXPHOS
Pathogen associated molecular patterns	PAMPs
Pathogen recognition receptors	PRRs
Parkinson's disease	PD
Peripheral blood mononuclear cells	PBMCs
Phorbol 12-myristate 13-acetate	PMA
Phosphatase and tensin homolog deleted on chromosome 10 induced kinase 1	PINK1
Phospholambin	PLN
Piperazine-*N*-*N'*bis[2-ethanesulfonic acid]	PIPES
Plasma membrane	PM

Polyethyleneimine	PEI
Primary progressive multiple sclerosis	PPMS
Principle component analysis	PCA
Prointerleukin-1beta	proIL-1β
PTEN-induced kinase 1	PINK1
Pyrin domain	PYD
Reactive oxygen species	ROS
Ryanodine receptors	RyRs
Sarcolipin	SLN
Sarco/endoplasmic reticulum calcium ATPase	SERCA
Single nucleotide variants	SNVs
SQSTM1	p62
Stimulator of interferon genes	STING
Three-dimensional	3D
TIR domain-containing adaptor protein inducing IFNβ	TRIF
Toll-like receptor	TLR
Transient receptor potential	TRP
Transient receptor potential melastatin-subfamily member 7	TRPM7
Tumor necrosis factor alpha	TNF-α
Vacuole membrane protein 1	VMP1
Voltage-dependent anion channels	VDACs

ABSTRACT

Inflammatory responses while beneficial when controlling infection need to be tightly regulated to prevent unnecessary damage to the host. Identifying novel regulators of inflammatory signaling is crucial to be able to fine-tune these responses during disease and mitigate unwanted responses under sterile or autoimmune conditions. One intriguing potential regulator of inflammatory signaling is vacuole membrane protein 1 (VMP1), an endoplasmic reticulum (ER)-resident protein known for its ability to regulate sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity and autophagy.

VMP1 was first characterized in acute pancreatitis models, an inflammatory disease that results in significant cell death. With connections to inflammatory disease, we sought to understand how VMP1 may regulate inflammatory signaling. For canonical inflammasome activation, two signals are required: the first signal induces upregulation of proinflammatory gene expression, and the second signal activates a multiprotein complex known as the inflammasome. Activation of these pathways ultimately results in the release of inflammatory mediators such as IL-6, IL-1β, and galectin-3 (gal-3). Evidence in the literature demonstrated that both damaged mitochondria and calcium fluxes can act as signals to activate these proinflammatory pathways. Given its known functions, VMP1 may regulate these responses by degrading damaged mitochondria and buffering cytoplasmic Ca^{2+} concentrations.

To address the regulatory role of VMP1 in inflammatory responses, differentiated THP-1s, a model macrophage-like cell line, were depleted of VMP1 using CRISPR-Cas9. VMP1 KO cells released more proinflammatory cytokines and gal-3 in response to inflammatory stimuli. VMP1 negatively regulated inflammasome activation in response to lipopolysaccharide (LPS) and ATP, canonical activators of the NLRP3 inflammasome. In response to inflammatory stimuli, VMP1 KO cells have elevated intracellular $[Ca^{2+}]$ that are buffered by mitochondria. This excess Ca^{2+} resulted in the loss of membrane potential of almost all the mitochondria and the release of cytoplasmic mitochondrial DNA (mtDNA). Exposed mtDNA likely acted as a damage associated molecular pattern (DAMP) to increase activation of the NLRP3 inflammasome. Additionally, in VMP1 KO cells there was impaired autophagic flux at the stage of autophagosome/lysosome fusion which likely prevented the degradation of damaged mitochondria. There was also increased cathepsin B activity which potentially also contributed to inflammasome activation. Collectively, these data demonstrated that VMP1 restricts NLRP3 inflammasome activation through its modulation of SERCA activity and autophagy. Decreased expression of VMP1 has been found in the peripheral blood mononuclear cells (PBMCs) of Parkinson's disease (PD) patients suggesting that diminished expression of VMP1 may be contributing to the inflammatory sequalae associated with the disease and perhaps can be targeted therapeutically in the future.

CHAPTER ONE

REVIEW OF THE LITERATURE

The Function of VMP1 in Cells.

VMP1 was first identified as an upregulated transcript in pancreatitis, but it is also

expressed in a variety of healthy tissues including brain, kidney, and intestine [9]. VMP1

is an ER-resident transmembrane protein that has been found to be essential for

autophagosome formation suggesting that it promotes homeostasis in the cell,

phagophore expansion and closure, lipid droplet formation, and is a crucial host factor

for SARS-CoV-2 and pan-coronavirus infection [10-13]. Related to these functions,

VMP1 has recently been identified to act as a phospholipid scramblase that facilitates

the normal cellular distribution of cholesterol and phosphatidylserine [14]. Interestingly,

VMP1 modulates contacts between the ER and mitochondria, lipid droplets, and

endosomes through its interaction with SERCA such that the interaction between VMP1

and SERCA reduces cytosolic Ca^{2+} levels close to these membrane contacts [5, 15].

While VMP1 has primarily been characterized so far for its role in autophagy,

studies in other organisms suggested that it may have diverse functions that include

regulating protein secretion, endocytosis, phagocytosis, and regulating organelle

function and morphology [16-19]. Surprisingly, in *Dictyostelium,* a versatile model

organism, the secretion of a protein, AprA, through the classical ER-Golgi network was

significantly released in wild type but attenuated from the VMP1 mutant (the VMP1 gene

was disrupted at amino acid 89) [16]. Phenotypes in this amoeba can be rescued by expressing mammalian VMP1 which suggested conservation of the function of this protein across species [16]. These findings were consistent with data showing that most autophagic structures colocalize with VMP1, but only 5% of VMP1 colocalizes with markers of autophagy [15]. Based on what is known so far, it seems incredible that one protein can regulate so many different cellular processes and the underlying mechanisms of this regulation are not yet well understood.

VMP1 and SERCA.

Recent work by Zhao et al. characterized a function for VMP1 in regulating the activity of SERCA, an ER-localized calcium channel that transports calcium from the cytoplasm into the ER lumen (Fig. 1) [5]. A mass spectrometry experiment revealed that VMP1 interacts with SERCA2, one of three major paralogs [5]. SERCA2 has differential Ca^{2+} affinity depending on its conformational state with the active form E1 conformation having high Ca^{2+} affinity and the E2 state having low Ca^{2+} affinity [20]. VMP1 acted as an activator of SERCA either by preventing formation of the SERCA/phospholambin (PLN)/sarcolipin (SLN) inhibitory complex or by stabilizing SERCA in its active form [21]. Interestingly, the interaction between SERCA and VMP1 modulates contact formation between the ER and mitochondria, lipid droplets, and endosomes [5]. The ER is connected to the PM, mitochondria, Golgi, and endosomes at membrane contact sites (MCSs) [22-24]. These ER contacts are important for mediating Ca^{2+} exchange, lipid transfer, and regulation of organelle dynamics such as fission, maturation, and

positioning [23, 24]. Formation of these contacts is dynamic and occurs in response to various stimuli. Global as well as local Ca^{2+} perturbations modulate ER contacts. Global cytosolic Ca^{2+} perturbations induce the ER-localized extended synaptotagmins to promote contacts between the ER and plasma membrane (PM) [25]. Due to the ability of VMP1 to modulate SERCA activity, VMP1 KO cells have decreased SERCA activity and an impaired ability to modulate changes in cytoplasmic $[Ca^{2+}]$.

Figure 1. VMP1 Interacts with SERCA. (Left) VMP1 interacts with SERCA to prevent the formation of the PLN/SLN inhibitory complex. Mitochondria (M), lipid droplets (LD), autophagosomes, and endosomes (E) can disassociate from the ER. (Right) In VMP1 KO cells, SERCA function is inhibited by formation of the PLN/SLN inhibitory complex which prevents the flow of Ca^{2+} from the cytoplasm to the ER to maintain low cytoplasmic $[Ca^{2+}]$. There is an increase in ER contacts with isolation membranes (IM), lipid droplets, mitochondria, and endosomes. PI(3)P contributes to enriched ER contacts in VMP1 KO cells. Reprinted from [5].

Of particular interest here is the fact that local Ca^{2+} release from ER channels prevents the motility of mitochondria and promotes their association with the ER [26]. Around 70% of mitochondria were in contact with the ER in VMP1 KO cells compared to

20% in control cells and tended to have predominately spherical and swollen morphology in KO cells [5]. VMP1 preferentially binds with SERCA in its active form, and loss of VMP1 expression results in a loss of Ca^{2+}-ATPase activity in the microsome fraction (mainly contains ER structures) compared to control cells which suggested that VMP1 promotes SERCA activity [5]. Proximity of VMP1 to SERCA competes with and prevents formation of the SERCA/PLN/SLN inhibitory complex. This inhibitory complex works by lowering the affinity of SERCA for cytosolic Ca^{2+} [27]. The interaction between VMP1 and SERCA reduces cytosolic Ca^{2+} levels close to these membrane contacts [5, 15]. Supporting the role for SERCA activity specifically in the persistence of membrane contacts is the fact that the presence of these ER contacts was increased by the non-competitive SERCA inhibitor, thapsigargin. Thapsigargin stabilized SERCA2 in its low Ca^{2+} affinity state, and the presence of thapsigargin interfered with the interaction between VMP1 and SERCA2 suggesting that VMP1 preferentially binds to the active form of SERCA2 [20]. ER contacts controlled by VMP1/SERCA are regulated by calmodulin (CaM), an intermediary protein that senses calcium levels and passes along signals to a number of calcium-sensitive enzymes, ion channels, and other proteins [5]. Overall, the interaction between VMP1 and SERCA serves to regulate the membrane contacts between the ER and various organelles by stabilizing SERCA in its active state.

VMP1 and Autophagy.

Autophagy is a cellular process that degrades and recycles cellular components. A portion of the cytosol is engulfed by a double-membrane autophagosome that is then

delivered to lysosomes for degradation (Fig. 2) [28, 29]. The initial step in autophagy induction is autophagosome formation. During this step, autophagosome precursors known as isolation membranes (IMs) have dynamic contact with the ER. Interestingly, it has been shown that in VMP1 KO cells there was stable association of IMs with the ER which prevented autophagosome formation [5].
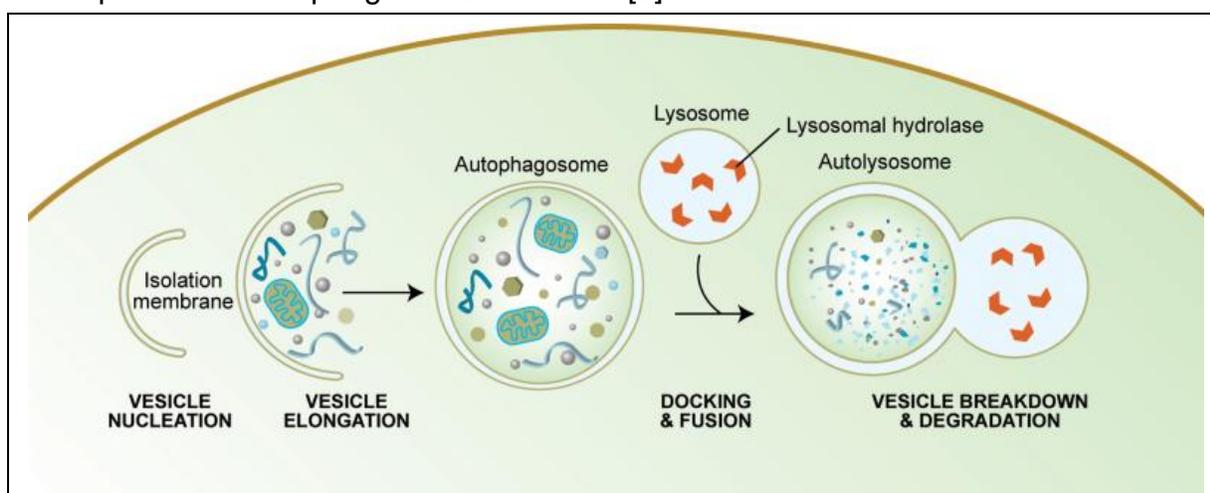
Figure 2. Overview of Autophagy. 1) Autophagy begins with the formation of the phagophore or isolation membrane (vesicle nucleation step). 2) Coordinated activity of autophagy core machinery proteins at the phagophore assembly site leads to the expansion of the phagophore into an autophagosome (vesicle elongation). The autophagosome can then engulf bulk cytoplasm nonspecifically, either specific cargo or entire organelles. 3) When the outer membrane of the autophagosome fuses with an endosome (forming an amphisome before fusing with the lysosome) or directly with a lysosome (docking and fusion steps), it forms an autophagolysosome. 4) The sequestered material is degraded inside the autophagolysosome (vesicle breakdown and degradation) and recycled. Reprinted from [2].

In addition to preventing autophagosome formation, VMP1 depletion also impaired autophagic flux at the step after LC3 lipidation evidenced by increased LC3II and p62 levels [5, 30]. Furthermore, in VMP1 KO cells, there was accumulation of LC3 puncta under both nutrient replete and starved conditions [5]. Some LC3 puncta were

larger than the ones in control cells. These LC3 puncta did not colocalize with LAMP1-labeled lysosomes suggesting that autophagic flux was impaired at the stage of autophagosome/lysosome fusion [5]. Additionally, in mice with the conditional KO of VMP1 in dopaminergic neurons, it was observed that there was accumulation of p62, more LC3 puncta, and less colocalization between LC3 and LAMP1 further supporting the idea that there was impaired autophagic flux in VMP1 KO cells [31]. Furthermore, an RFP-GFP-LC3 tandem reporter was used to determine the stage of autophagy of the LC3 puncta. In this construct, the GFP signal was quenched in acidic environments such that yellow vesicles were representative of IMs and immature autophagosomes and the red ones were acidified mature autophagosomes and autolysosomes [32]. Further supporting that autophagy was reduced in VMP1 KO cells, all of the LC3 puncta were yellow [5]. In VMP1 KO cells, disassociation of LC3-labeled autophagic structures from the ER was defective [5]. Overall, in VMP1 KO cells, there was compromised autophagosome formation and autophagic flux was stalled prior to autophagosome/lysosome fusion.

<center>**VMP1 Expression Levels and Disease.**</center>

VMP1 and Cancer.

In the last few years, altered VMP1 expression is increasingly being identified for its connection to disease. As the association between VMP1 expression levels and disease is better understood, measuring VMP1 expression levels could have potential prognostic value. Overexpression of VMP1 was associated with advanced glioma, an aggressive type of brain tumor, and poor prognosis [33]. Knockout of VMP1 expression

sensitized glioma cells to radiotherapy and chemotherapy. A model showed that VMP1 expression levels has prognostic value for determining survival in glioma patients [33]. Similarly, studies that measured VMP1 expression in patients with ovarian cancer and acute myeloid leukemia found that elevated VMP1 expression contributed to cancer progression and correlated with a poor prognosis [34, 35]. In contrast, in hepatocellular carcinoma and colorectal cancer, low VMP1 expression correlated with advanced cancer stage and shorter survival [36, 37]. Collectively, VMP1 expression levels were correlated with a variety of cancers, but whether increased or decreased expression correlated with cancer development and progression depended on the type of cancer. Yet what is known so far is that the link between VMP1 and cancer seemed to be related to the ability of VMP1 to regulate autophagy [33, 35, 37].

VMP1 and Parkinson's Disease.

Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta accompanied by the accumulation of intracytoplasmic α-synuclein (α-syn) containing Lewy bodies [38, 39]. Expression of VMP1 was assessed in the PBMCs isolated from a cohort of PD patients [40]. In general, the PD group had significantly lower VMP1 expression compared to a healthy control cohort [40]. This disease affects 1% of the population over the age of 60 [38, 39]. Death of DAergic neurons affects dopamine transmission which results in the primary motor symptoms of the disease. These include tremor at rest, rigidity, and postural instability [41, 42]. Disease onset occurs decades before the first symptoms appear. Only about 5-10% of PD cases are associated with genetic

predisposition. Familial cases of PD can be caused by mutations in genes that encode for the proteins, α-syn, DJ-1, PINK, LRRK2, etc. However, most cases are idiopathic, but there tends to be increased risk with age. Other risk factors include exposure to environmental toxins, pesticides, heavy metals, traumatic lesions, and bacterial or viral infections which interestingly are all associated with inflammation [43].

Inflammatory responses in both the periphery and the CNS contribute to PD disease pathogenesis (Fig. 3). The "gut-brain axis" hypothesis suggests that dysregulation in the gut can promote α-syn aggregation and inflammation in the periphery. This hypothesis was supported by elevated cytokine levels, including IL-1β, IL-2, IL-6, IFN-γ, and TNF-α and increased CD4+ lymphocyte counts in the serum and cerebrospinal fluid (CSF) of PD patients supporting this idea for the presence of peripheral inflammation [44, 45]. It was then postulated that accumulation of α-syn in the intestine can spread through the vagus nerve to the brain in a fashion that mimics a prion-like disease [46]. Ultimately, glial cells in the CNS are activated by PAMPs and DAMPs secreted from damaged neurons or protein aggregates. This response results in chronic neuroinflammation that is thought to be critical to disease progression [47].

A recent study identified that there was significantly lower VMP1 expression in the PBMCs of a cohort of PD patients compared to a healthy control group [40]. In these patients, decreased VMP1 expression correlated with the length and severity of disease [40]. This decrease in VMP1 expression in PD patients could be ameliorated by the administration of a DAergic receptor agonist monotherapy or a combination of a

DAergic receptor agonist and L-dopa [40]. Furthermore, a recent study found that there was increased expression of the proinflammatory NLRP3 and procapase-1 genes in the PBMCs of PD patients as well as increased protein levels of NLRP3, caspase-1, and IL-1β in the blood plasma which correlated with disease severity. Levels of IL-1β and IL-6 also were elevated in the CSF of PD patients [48]. Interestingly, it has been found that the peripheral monocytes of PD patients have a hyperactive response to LPS [49]. These data support that PD is in fact a chronic systemic inflammatory disease and that there are likely novel regulators, potentially VMP1, that can attenuate inflammatory responses during the course of this disease [50].

In PD patients, pathological α-syn aggregates can act as a DAMP to trigger proinflammatory responses in the CNS [51-53]. Several studies have corroborating evidence that several toll-like receptors (TLRs), namely TLR2, TLR4, and TLR9, were involved prominently in the pathogenesis of PD [54, 55]. Oligomeric α-syn released from neuronal cells can act as a ligand for TLR2 expressed on microglia to induce an inflammatory response [56]. Furthermore, when TLR2 was activated, it has been observed that activation of this pathway can promote endogenous α-syn aggregation [57]. Studies of human postmortem brain showed that there was increased expression of TLR4 and its adaptor protein MyD88 particularly in the substantia nigra and putamen of PD patients [58-60]. Hughes et al. showed that oligomeric α-syn induced TNF-α production in WT mice at levels that were 10 to 100 times greater than in TLR4 knockout mice, yet the effect in TLR2 knockout mice was more modest suggesting that TLR4 was likely the primary TLR that recognized α-syn and initiated an inflammatory

response [59]. Collectively in the literature, the role of TLR4 seemed to vary depending

on the stage of disease progression.

Figure 3. Inflammation in PD. Both central and peripheral inflammation contribute to PD progression and pathogenesis. The "gut-brain axis" hypothesis in PD postulates that changes in the gut microbiome may lead to α-synuclein aggregation and an inflammatory response in the periphery which includes increased cytokine levels and activated T cells. Aggregated α-syn is then thought to spread from the periphery to the brain through the vagus nerve in a prion-like manner. Once in the brain, proteinopathy and other proinflammatory factors (mitochondrial impairment, ROS, etc.) will cause and sustain central inflammation in a cycle between dying dopaminergic neurons, glial cells and activated endothelium. Central inflammation will be aggravated by infiltrating peripheral immune cells. Reprinted from [3].

For example, in the acute phase of disease, TLR4 recognized α-syn causing it to be

phagocytosed and cleared to delay disease progression. However, in the chronic phase

of PD, excessive stimulation of TLR4 resulted in proinflammatory cytokine production causing neuroinflammation and ultimately neurodegeneration which contributed to disease progression [61]. Supporting the "gut-brain" hypothesis were data demonstrating that there was higher TLR4 expression in the colonic samples of PD patients compared to control. In accordance with this higher expression, it was observed that there was disruption of the intestinal barrier, increased microbial markers, and a more proinflammatory gene profile in the colonic samples from these patients [62]. At this point, numerous studies have demonstrated that TLR signaling responses can be activated to contribute to PD pathology.

Prior work by Wang et al. utilized a mouse model with a conditional knockout of VMP1 in DAergic neurons, one of the key cell types involved in PD [31]. In this study, it was observed that in mice with conditional VMP1 KO in DAergic neurons there were motor defects, severe loss of DAergic neurons, and disturbance of autophagic flux [31]. In these mice, there was accumulation of α-syn in the striata suggesting that VMP1 expression may help to prevent misfolded protein aggregation which ultimately results in neuronal loss [31]. As mentioned, one of the hallmarks of PD pathology was the presence of α-syn aggregates. Clearance of α-syn aggregates by autophagy was dependent on p62/SQSTM1, a protein that binds polyubiquitinated proteins and can act as a scaffold for autophagic machinery by directly interacting with polyubiquitin and LC3 [63-66]. Previous work in *Dictyostelium*, a model organism often used to investigate human genes associated with disease and host/pathogen interactions, demonstrated

that VMP1 may help to mediate the clearance of α-syn aggregates in PD [67].

Dictyostelium have many conserved genes with humans including VMP1 [16]. In

Dictyostelium vmp1⁻ mutant cells had an accumulation of large ubiquitin-positive protein

aggregations that included the autophagy marker GFP-Atg8 and the putative

Dictyostelium p62 homologue [68]. Reduced colocalization between ubiquitin-positive

aggregates and lysosomes suggested that VMP1 was required for the clearance of

ubiquitinated protein aggregates [68]. Given that it has been observed that VMP1

promotes autophagic flux, decreased expression of VMP1 in PD patients would likely

contribute to the accumulation of aggregated α-syn characteristic of disease pathology,

but the function of VMP1 has not yet been studied in macrophages.

VMP1 and Multiple Sclerosis.

Several studies have also linked changes in the VMP1 gene and its expression

to multiple sclerosis (MS). MS has a strong yet complex genetic component, and

several studies have identified single nucleotide variants (SNVs) in the VMP1 gene

associated with MS [69-71]. MS, an autoimmune disease of the CNS, has a

heterogenous clinical presentation that has been characterized by demyelination and

formation of neurological lesions [72]. Historically, the disease has been thought to have

a complex etiology likely due to a combination of genetic and environmental risk factors,

but in the last year, it has been identified that Epstein-Barr Virus infection is likely the

leading cause of MS [71-73]. The onset and development of MS has been characterized

by multiple inflammatory events (Fig. 4).

Figure 4. Mechanism of Disease Pathogenesis in MS. At the onset of disease, autoreactive CD4+ T cells are activated in the periphery by antigen presenting cells. These cells present antigen in the context of MHC class II that look like proteins synthesized in the CNS. 1) Antigen presentation activates the differentiation of naïve CD4+ T cells into CD4+ T helper cells. 2) Upon activation, T helper cells produce IFN-γ, a cytokine that recruits CD8+ T cells, B cells, and monocytes. 3) These proinflammatory cells migrate to the CNS where they can cross the blood brain barrier. In the brain, plasma B cells produce antibodies against CNS self-antigens resulting in damage to the myelin sheath. Infiltrated cytotoxic CD8+ T cells attack and destroy oligodendrocytes and cause neuronal cell death. Monocytes increase local inflammatory responses by releasing proinflammatory cytokines and contribute to demyelination through myelin phagocytosis. 4) At the same time, infiltrated CD4+ T cells are reactivated when they interact with myelin fragments that are presented by the resident APCs. This results in 5) the release of proinflammatory cytokines and chemokines and 6) astrogliosis and microgliosis which are defense mechanisms by resident CNS immune cells in response to damage. Reprinted from [7].

First, starting in the periphery, antigen presenting cells (APCs) presented peptides with similar structures to myelin found in the CNS via class II major histocompatibility complexes (MHC) to activate autoreactive CD4^{+} T cells [74]. Following this interaction, naïve CD4^{+} T cells differentiated into CD4^{+} T helper cells [75]. When these cells were activated, the Th1 subtype produced interferon gamma (IFN-γ), a cytokine that recruits CD8^{+} T cells, B cells, and monocytes to the periphery [76, 77]. These proinflammatory cells then can migrate through the bloodstream to the blood brain barrier (BBB) where they adhere to the endothelial cells [78]. In individuals suffering from MS, there was altered expression and organization of endothelial tight junctions that allowed for massive infiltration of immune cells into the brain [79, 80]. CD4^{+} T cells that infiltrated into the CNS were reactivated by the resident APCs [81]. The reactivated cells then released proinflammatory cytokines and chemokines that caused astrogliosis and microgliosis [82-84]. The damage was augmented by infiltrating CD8^{+} T cells that attack oligodendrocytes, destroying them and causing neuronal cell death [85]. At the same time, plasma B cells produced antibodies against self-antigens in the CNS which contributed to myelin sheath damage [86]. Plasma B cells along with monocytes exacerbated the local inflammatory response by reactivating the autoreactive CD4^{+} T cells [87]. Given that MS was characterized by a cascade of inflammatory events, it was likely that environmental and genetic risk factors that promote inflammatory responses likely played a key role in the development of this disease.

Many studies have been done to identify SNVs in the MS population. One of the goals of the International Multiple Sclerosis Genetics Consortium was to analyze the genetic data of MS and control subjects to create a reference map of variants in the MS population. Most of the susceptibility variants have been found in the MHC as expected given that it has been identified as an important contributor to disease pathology. This study prioritized about 500 genes outside of this region with most of the genes being related to the immune response [70]. The results of this analysis identified a SNV in the VMP1 gene. This change was located on chromosome 17 at base pair 59781849 with the base pair change of G to A (the SNP ID is rs2150879) [70]. The allele frequency was between 0.5-0.9 in different populations [70]. Other studies have focused on analyzing datasets from individuals in Europe given that the development of MS has been most prevalent in Northern Europeans between 0.1-0.2% of the population [72]. Three bioinformatics analyses found an association between disease and individuals that have a different SNV in the VMP1 gene located on chromosome 17 at base pair 59739396 with the base change of T to C (the SNP ID is rs8070345) [69, 71, 88]. The two SNVs identified in these studies were intronic which made it less clear how these SNVs might affect the amount of VMP1 protein. Additionally, the study that analyzed data from an Italian population identified 141 SNVs in the VMP1 gene [69-71]. These genetic analyzes together suggested that there was an increased frequency of SNVs in the VMP1 gene in people that develop MS. Furthermore, one study identified changes in the methylation of the *VMP1/MIR21* locus in CD4+ T cells [89]. Individuals with relapsing/remitting MS had higher methylation of the last two exons of these genes in

CD4$^+$ T cells than individuals with secondary-progressive MS disease and healthy controls. These methylation changes seemed to not be correlated with known MS risk variants in *VMP1* [89]. Perhaps the most compelling data linking VMP1 and MS was a recent study that used single cell RNA-sequencing to analyze gene expression changes in MS patients' cells. It was observed that there was decreased expression of VMP1 in the monocytes of patients with primary progressive multiple sclerosis (PPMS), a form of the disease that was characterized by a progressively worsening condition [90]. Overall, these studies suggested that there were changes in the VMP1 gene in MS patients compared to healthy controls that would need to be further studied.

Increasing evidence suggested that inflammasome activation played a critical role in MS pathogenesis [91]. Several studies have found that caspase-1 mRNA levels were elevated in the white matter of MS patients compared to control groups and that it was overexpressed in MS plaques, infiltrating perivascular mononuclear cells and parenchymal macrophages/microglia [92]. Another study showed that the expression of NLRP3, ASC, caspase-1, and IL-1β was increased in reactive astrocytes and infiltrating macrophages/microglia of active demyelinating lesions of MS, but it was decreased in chronic inactive lesions of MS [93]. The inflammasome can potentially be used as a diagnostic biomarker with a cohort study showing higher levels of caspase-1 and ASC in the serum of MS patients. Specifically, ASC levels could differentiate disease severity with levels being higher in moderate MS patients compared to mild MS patients [94]. Furthermore, a recent study showed that PPMS patients (who have decreased

expression of VMP1 in their monocytes) have higher levels of NLRP3 compared to relapse-onset MS and healthy control subjects [90, 95]. Interestingly, when mice with experimental autoimmune encephalomyelitis, a common MS mouse model, were treated with the small molecule inhibitor of caspase-1, VX-765, there was reduced expression of inflammasome- and pyroptosis-associated proteins in the CNS, reduced axonal injury, and improved neurobehavioral performance suggesting a role for caspase-1 activation in MS pathogenesis [96]. Future work would need to characterize specifically the function of VMP1 in MS, but perhaps decreased VMP1 expression exacerbates the inflammatory responses associated with the disease.

Inflammation and Disease.

Innate immune cells such as macrophages play a critical role in the initial recognition of potential danger and mounting a response to protect against it. These cells express pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) or signals indicating cellular stress termed damage associated molecular patterns (DAMPs) [97]. Unfortunately, if this response is not tightly regulated, there will be unnecessary or excessive inflammatory responses resulting in disease. Some of these inflammatory diseases include cryopyrin-associated periodic syndrome, arthritis, atherosclerosis, type 2 diabetes, Alzheimer's disease (AD), PD, MS, and cancers [98, 99].

Toll-like Receptor Signaling.

TLRs are the first line of defense for the immune system. TLRs are part of a family of PRRs [100]. They recognize molecules from both invading pathogens known

as PAMPs and endogenous danger signals released from dying cells known as DAMPs [101, 102]. Activation of TLRs leads to downstream signaling cascades that act to defend the host against invaders or to repair damaged tissue. These responses are mediated by the release of inflammatory cytokines and immune modulators [103]. The first TLR that was identified in humans was TLR4 which upon activation was shown to upregulate the expression of genes involved in inflammatory responses [104]. TLRs are expressed on the cell surface (heterodimers of TLR2 with TLR1 and TLR6 in addition to TLR4, TLR5, and TLR10) and inside of the cell either on the ER, endosomes, and lysosomes (TLR3, TLR7, TLR8, and TLR9) (Fig. 5) [1]. Of particular interest here are TLR4 and TLR3. TLR4 is activated by LPS found on the surface of gram-negative bacteria [105]. TLR3 is activated by double-stranded RNA (dsRNA) from viruses [106].

Downstream of TLR activation are adaptor proteins either myeloid differentiation primary response protein 88 (MyD88) for TLR4 or TIR domain-containing adaptor protein inducing IFNβ (TRIF) which can act independently of MyD88 following TLR4 or TLR3 activation [107, 108]. This signaling results in activation of the transcription factor, NF-κB, which leads to the upregulation of proinflammatory gene expression [109, 110]. Some of these proinflammatory cytokines include tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and prointerleukin-1β (proIL-1β) [111]. Ultimately, either proinflammatory cytokines or type I interferon are produced depending on the stimulus along with downstream signaling to promote the cellular response to invading pathogens [101].
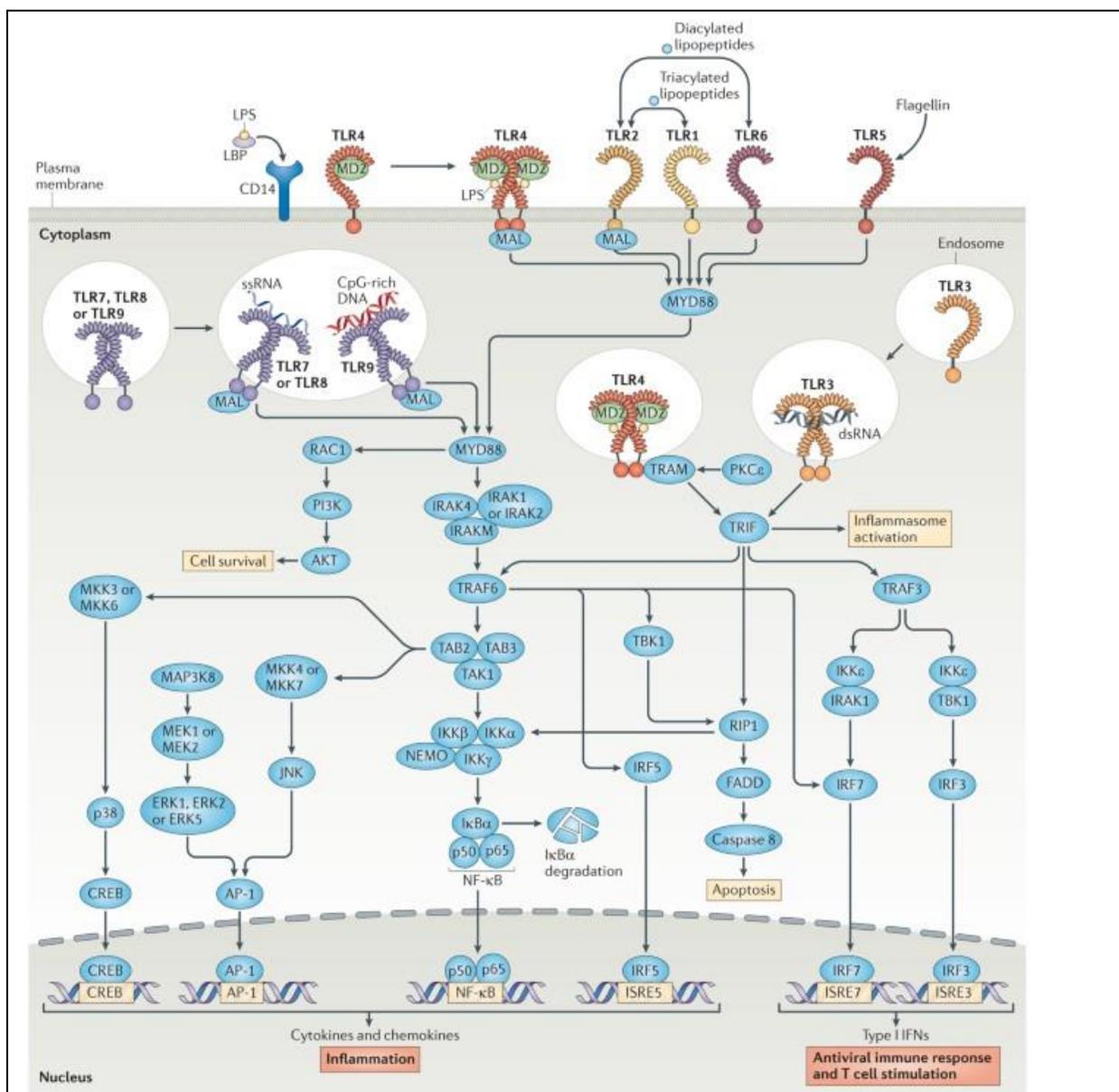
Figure 5. Overview of TLR Signaling Pathways. TLRs are expressed on the cell surface and in endosomes. They detect microbial cell-wall components, non-self nucleic acids or danger-associated self-molecules. Upon stimulation, TLRs activate two types of pathways that involve either MYD88 or TRIF. Crosstalk with other signaling pathways ensures that the TLR signal is properly regulated and leads to either apoptosis or cell survival, and the transcription of proinflammatory cytokines and chemokines and type I interferons. Reprinted from [1].

Cytokines are short-lived but can travel long distances to act on a variety of tissues and elicit a systemic response.

Canonical NLRP3 Inflammasome Signaling.

In the canonical NLRP3 inflammasome pathway, two signals are required: the first signal induces upregulation of proinflammatory gene expression mediated by TLR activation, and the second signal activates a multiprotein complex specifically the NLRP3 inflammasome. Activation of these pathways ultimately results in the release of inflammatory mediators such as IL-6, interleukin-1β (IL-1β), and gal-3. Inflammasome activation is a key component of the innate immune response and is modeled in Fig. 6 (left). The inflammasome consists of a ligand-sensing protein such as nucleotide-binding domain, leucine-rich repeats containing family, pyrin domain-containing-3 (NLRP3), an adaptor protein such as apoptosis-associated speck-like protein containing a CARD (ASC), and finally an effector protein such as caspase-1 [112, 113].

The key PRR of interest here is the cytoplasmic sensor, NLRP3, which is part of the NOD-like receptor (NLR) family. More specifically, NLRP3 has three domains: a carboxy-terminal leucine-rich repeat (LRR) that can recognize signals and has autoinhibitory functions, a central nucleotide-binding domain (NACHT) that mediates self-oligomerization and has ATPase activity, and an amino-terminal pyrin domain (PYD) needed to recruit ASC [114]. One thing that is unique about the NLRP3 inflammasome is that it is activated in response to a variety of stimuli including PAMPs such as viral and microbial components and DAMPs including ATP, uric acid crystals, and β-amyloid peptides. These signals induce K^+ efflux, Cl^- efflux, mitochondrial

dysfunction, ROS release, mtDNA production, and lysosomal disruption to assemble and activate the NLRP3 inflammasome. NLRP3 senses these signals and self-oligomerizes via interactions between homotypic NACHT domains. Oligomerization of NLRP3 results in the recruitment of ASC through homotypic PYD-PYD domain interactions. This interaction results in the formation of a macromolecular complex with multiple copies of ASC, known as an ASC speck [115]. Following recruitment of ASC, this assembly recruits procaspase-1 through interactions between the homotypic CARD-CARD domains.
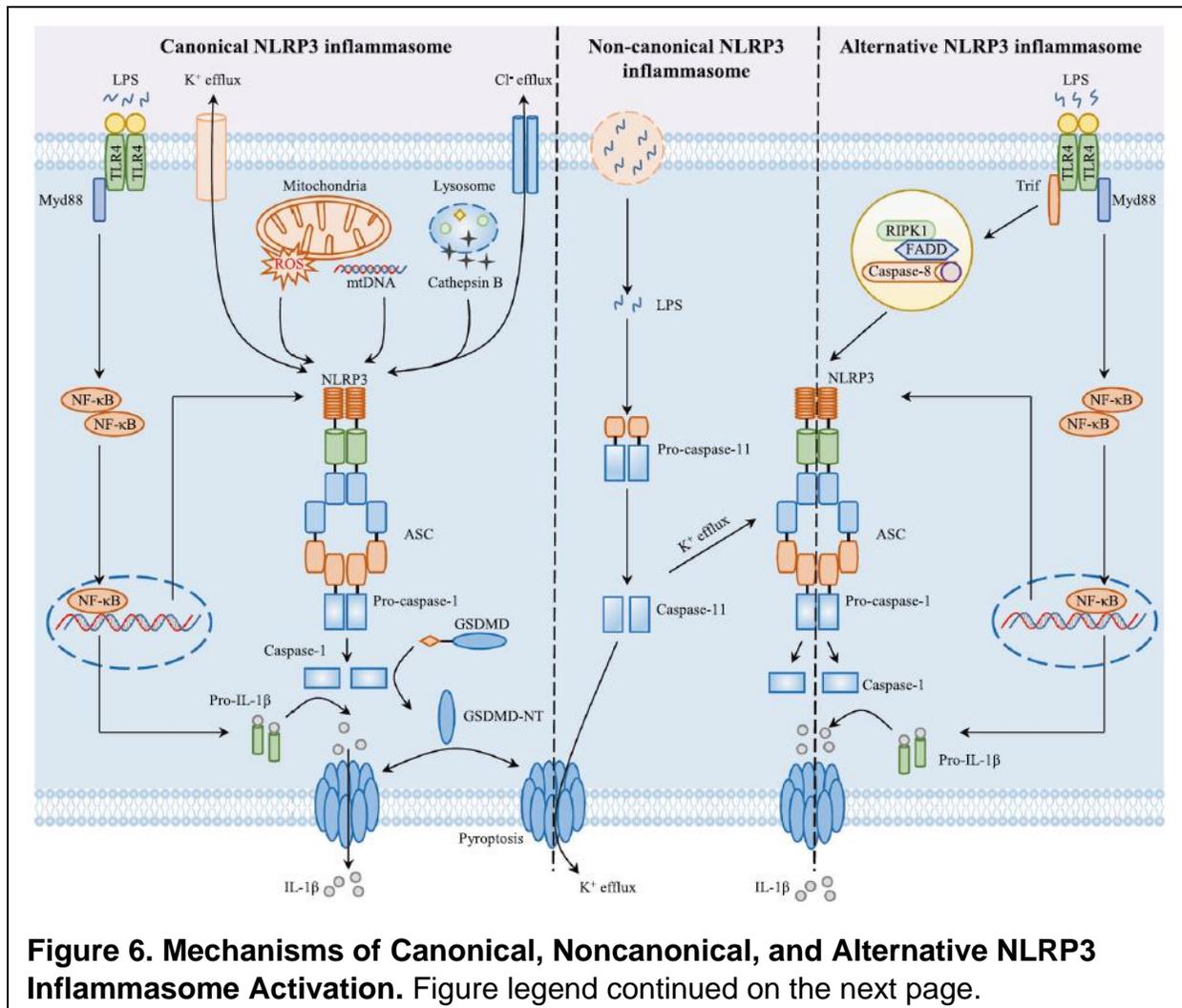
Figure 6. Mechanisms of Canonical, Noncanonical, and Alternative NLRP3 Inflammasome Activation. Figure legend continued on the next page.

Figure 6. Mechanisms of Canonical, Noncanonical, and Alternative NLRP3 Inflammasome Activation. (Left) Canonical NLRP3 inflammasome activation requires two signals: the priming signal and the activation signal. During the priming step, TLRs, NLRs, and cytokine receptors induce activation of NF-κB to upregulate the expression of proIL-1β and NLRP3. During the activation step, a variety of PAMPs and DAMPs induce K^+ efflux, Cl^- efflux, mitochondrial dysfunction, ROS release, mtDNA production, and lysosomal disruption to assemble and activate the NLRP3 inflammasome. Procaspase-1 then self-cleaves into its active form. It then cleaves proIL-1β and proIL-18 into their mature forms allowing them to be released from the cell, and it cleaves GSDMD. The N-terminal domain of GSDMD forms pores in the PM to mediate the release of proinflammatory cytokines and induce an inflammatory form of cell death termed pyroptosis. (Middle) Cytosolic LPS is sensed by caspase-11 (in mice) and caspase-4/-5 (in humans) to initiate noncanonical NLRP3 inflammasome activation. Caspase-11 is activated by autoproteolysis. Active caspase-11 opens the pannexin-1 channel to induce K^+ efflux to activate the canonical NLRP3 inflammasome. At the same time, caspase-11 cleaves GSDMD to induce membrane pore formation and pyroptosis. (Right) Alternative NLRP3 inflammasome activation only requires one signal. TLR ligands alone can activate the NLRP3 inflammasome in human and porcine monocytes via the TLR4-TRIF-RIPK1-FADD-CASP8 signaling axis which does not induce K^+ efflux, ASC speck formation, or pyroptosis but does induce the release of IL-1β through a noncanonical secretion pathway. Modified from [8].

The complex containing NLRP3, ASC, and caspase-1 is known as the NLRP3 inflammasome [116]. After the inflammasome is activated, procaspase-1 self-cleaves into its active form leading to the cleavage and maturation of the proinflammatory cytokines, IL-1β and interleukin 18 (IL-18). Caspase-1 also cleaves gasdermin D (GSDMD), the N-terminal domain of which forms pores in the PM leading to the release of the proinflammatory cytokines, IL-1β and IL-18, and induction of a type of inflammatory cell death known as pyroptosis [117-119].

The primary focus of this work is on the canonical inflammasome pathway, but it also has been recently identified that there are mechanisms of noncanonical and

alternative NLRP3 inflammasome activation (Fig. 6) (middle) (right). Briefly, in noncanonical inflammasome activation, cytosolic LPS is sensed by caspase-4/-5 in humans. Once activated by autoproteolysis, caspase-4/-5 opens the pannexin-1 channel to induce K^+ efflux and activate the NLRP3 inflammasome. Caspase-4/-5 can also then cleave GSDMD to form membrane pores and induce pyroptosis. In alternative inflammasome activation, only one signal is required in human monocytes. TLR ligands alone can activate the TLR4-TRIF-RIPK1-FADD-CASP8 signaling axis to induce the release of IL-1β through a nonconventional secretion mechanism [8].

Gal-3 and Unconventional Protein Secretion.

In addition to the proinflammatory cytokines already mentioned, gal-3 is an inflammatory β-galactoside-binding lectin that also is secreted from activated macrophages. Galectins are a family of proteins that positively contribute to immunity and development but can negatively influence cancer and inflammation [120-123]. Gal-3 is a unique member of the galectin family in that it has an approximately 120 amino acid N-terminal domain of tandem repeats (proline, glycine, and tyrosine), a region that is susceptible to cleavage by metalloproteinases that is connected to a C-terminal CRD domain that can bind β-galactosides as well as the NWGR anti-death motif which is highly conserved within the Bcl-2 protein family [122, 124-126]. Gal-3 is post-translationally modified at Ser6 and Ser12 located in the N terminal domain which can modulate its ability to bind to carbohydrate domains and signal for its nuclear localization and oligomerization [125-127]. Purified gal-3 can associate in monomers, dimers, or oligomers in the absence of ligand binding and the protein can self-

oligomerize through the N-terminal domain [128, 129]. Following interaction with its ligand, there is cooperativity in the N-terminal domain of gal-3 that facilitates the recruitment of other gal-3 polypeptides [128, 130]. Gal-3 can be released from cells through several different mechanisms. Since gal-3 lacks a conventional leader signal sequence for secretion, it is thought that the N-terminal domain directs its localization to secretory vesicles [131, 132]. Gal-3 has been found localized in microdomains in the PM and can directly pass through the lipid bilayer through interactions with cholesterol and phospholipids [133]. Our data presented here and other previous work suggested that under inflammatory conditions, gal-3 is released through GSDMD pores [134].

Characterizing how gal-3 is released from cells is important for understanding its role in inflammatory responses and disease. Gal-3 is known to be upregulated in several neurodegenerative disease models [135-140]. Only a subset of galectins including gal-3 are known to be expressed in the brain, but their function is not yet well-understood [141, 142]. In microglia, there is minimal gal-3 expression at basal levels, but it can be upregulated by inflammatory signals [135]. Gal-3 has been found to contribute to both pro- and anti-inflammatory phenotypes primarily in microglia and oligodendrocytes in the brain, yet the underlying mechanisms of these functions are still ambiguous. It seems to depend on the type of injury, stage of disease/trauma, and timing of intervention [140, 141, 143-145]. Notably, in patients suffering from Huntington's disease, a neurodegenerative disease, plasma levels of gal-3 correlated with disease severity. Gal-3 expression levels also have been found to be elevated in brain sections from AD patients [141, 146, 147]. In this disease model, all gal-3 puncta

were detected in Iba-1 positive cells suggesting localization to microglia and not other cell types [141]. Prior work has found that LPS-treated microglial cells release gal-3 which can then promote inflammatory responses by signaling through TLR4 expressed by neighboring cells [135]. In contrast, another study found that gal-3 released from macrophages can negatively regulate LPS-induced inflammation by binding to LPS which prevents LPS from activating TLR4 [148]. When reviewing the literature, gal-3 has both pro- and anti-inflammatory effects in the brain that will warrant additional research. Understanding how gal-3 is released from microglia in the brain under various inflammatory conditions may allow for gal-3 to be targeted therapeutically to perhaps minimize the propagation of inflammatory responses that contribute to neurological disease development and progression.

Regulation of Inflammatory Signaling.

Inflammatory responses while beneficial when controlling infection need to be tightly regulated to prevent unnecessary damage to the host. Identifying novel regulators of inflammatory signaling is crucial to be able to fine-tune these responses during disease and mitigate unwanted responses under sterile or autoimmune conditions. Several autoinflammatory diseases are caused by the dysregulation of inflammasome activation resulting in high levels of secreted IL-1β and/or IL-18, and we hypothesize that cells expressing decreased levels of VMP1 will exhibit dysregulated inflammatory responses [149]. In the following sections, mechanisms that regulate inflammatory signaling will be reviewed with particular emphasis on those mechanisms that are likely influenced by VMP1. VMP1 likely regulates inflammatory responses due

to its ability to regulate membrane contacts, calcium signaling, and mitochondrial function [5, 150].

Regulation of Toll-like Receptor Signaling.

TLRs respond to a variety of structurally conserved molecules derived from microbes and as such these responses are regulated by several mechanisms. Several TLRs recognize nucleic acids including TLR3 which recognizes dsRNA; TLR7, TLR8, and TLR13 recognize fragments of single-stranded RNA with distinct sequence preferences; and TLR9 recognizes single-stranded DNA containing unmethylated CpG motifs. Collectively, these nucleic acid-sensing (NA-sensing) TLRs are particularly important for the response to viral infections but come with the risk of potentially sensing self-nucleic acids [151]. Improper activation of NA-sensing TLRs by self-nucleic acids has been associated with autoimmune and autoinflammatory diseases, including systemic lupus erythematosus and psoriasis [152-158]. Each TLR has a threshold that must be met for activation with each likely having distinct mechanisms of regulation. For these TLRs, the primary mechanisms of regulation include intracellular compartmentalization, ligand availability, receptor expression, and signal transduction. Compartmentalization of these TLRs to the endosome physically separates them from self-nucleic acids although it has been shown that these receptors can be activated if the ligands are specifically delivered to the endosomes [157, 159]. In contrast, the cGAS-STING pathway also recognizes DNA, but these sensors are localized in the cytoplasm where they can also sense self-nucleic acids [160]. Currently, there is a lack of information regarding how TLR expression is regulated, yet several studies have

identified increased expression of TLR3 and TLR4 to be associated with disease [161-164]. TLR activation can also be regulated at the level of signal transduction which will be outlined in the next section.

TLR Activation and Calcium Signaling.

Ca^{2+} is a second messenger molecule in several cellular processes. Changes in $[Ca^{2+}]$ also controls signal transduction downstream of TLRs (Fig. 7) [6]. The cytosolic levels of Ca^{2+} are regulated by the activity of several organelles with the ER and mitochondria being of particular interest here. The Ca^{2+}-ATPase, SERCA, transports Ca^{2+} from the cytoplasm to the ER. VMP1 regulates the activity of SERCA which by extension suggests that VMP1 can regulate Ca^{2+} fluxes under inflammatory conditions as described in this work. In opposition, Ca^{2+} is released from the ER via inositol 1,4,5-trisphosphate receptor channels (IP3Rs) and ryanodine receptors (RyRs). Mitochondria take up Ca^{2+} through the mitochondrial Ca^{2+} uniporter (MCU) complex and the voltage-dependent anion channels (VDACs). Mitochondria extrude Ca^{2+} into the cytoplasm through the mitochondrial NCX (mNCX) and the mitochondrial permeability transition pore (mPTP) [165-167]. The activity of these various channels helps to regulate cytoplasmic $[Ca^{2+}]$ which tend to be very low which creates an environment that is sensitive to changes in calcium.

Transient receptor potential (TRP) channels are a family of ion channels that mediate the movement of different cations, especially Ca^{2+}, across the plasma membrane and into the cell.

Figure 7. Crosstalk between TLRs and Ca²⁺ Signaling. Activation of TLR2 sensitizes PLC to produce IP3 which activates IP3R to cause Ca²⁺ release from the ER. Ca²⁺ release from the ER promotes TLR2 activation. TLR2 activation upregulates ORA1 and STIM1 expression which increases Ca²⁺ entry. Activation of TLR3 triggers Ca²⁺ release from the ER through IP3R. Activation of TLR3 also upregulates expression of ORAI1 and STIM1. Ca²⁺ signaling increases TLR3 expression and regulates TLR3 activation via S100A9. Activation of TLR4 induces the influx of Ca²⁺ through TRPC1, TRPC3, TRPC6, TRPV1, TRPV2, TRPV4, TRPM3, TRPM7, and TRPM8 on the cell membrane and efflux through NCX1. Upon LPS stimulation, TLR4 promotes Ca²⁺ release from the ER through PLC to stimulate IP3 production and activate IP3R. RyR also contributes to the release of Ca²⁺ from the ER following activation of TLR4. Activation of TLR4 also upregulates STIM1 and ORAI1 expression. Increased intracellular Ca²⁺ promotes TLR4 activation. Depending on the extracellular [Ca²⁺], MBL can inhibit TLR4 activation. TLR7 activates PKA to activate SERCA and RyR2 located on the ER. TLR7 induces the IP3-dependent release of Ca²⁺ from the ER through IP3R. The cell membrane-resident Ca²⁺ channel Mcoln1 promotes TLR7 mediated immune responses. TLR8 activation increases cytosolic levels of Ca²⁺. TLR9 stimulation reduces the activity of SERCA2 located on the ER in cardiomyocytes. TLR9 activation induces Ca²⁺ release from the ER and sensitizes STIM1 to facilitate Ca²⁺ entry. IP3: inositol-1,4,5-triphosphage, ER: endoplasmic reticulum, IMQ: imiquimod, IP3R: IP3 receptor. Reprinted from [6].

At least nine different members of this family of ion channels have been reported to be involved in increases in cytosolic Ca^{2+} levels induced by LPS activation of TLR4 [168-174]. Work using bone marrow-derived macrophages (BMDMs) demonstrated that the transient receptor potential melastatin-like 7 (TRPM7), a non-selective but Ca^{2+}-conducting ion channel, mediated the cytosolic Ca^{2+} elevations essential for LPS-induced macrophage activation [174]. Data suggested that TRPM7-dependent Ca^{2+} elevations were essential for the nuclear translocation of NF-κB and that TRPM7-deficient macrophages were not able to produce IL-1β and other proinflammatory cytokines [174]. Also, the ligands for TLR3, TLR4, and TLR9 induce calcium fluxes and activate calcium/calmodulin-dependent protein kinase II (CaMKII) in macrophages [175]. CaMKII-α can activate the mitogen-activated protein kinase (MAPK) and NF-κB pathways. Selective inhibition or RNA interference of CaMKII suppressed the production of IL-6, TNF-α, and IFNα/β in macrophages. This study demonstrated that the cross-talk between intracellular calcium fluxes and the CaMKII pathway were needed for activation of TLR signaling in macrophages [175]. Overall, these data demonstrated that calcium fluxes were essential to produce proinflammatory cytokines downstream of TLR signaling.

The Role of Ca^{2+} Signaling on NF-κB Activation.

NF-κB is a transcription factor that is activated downstream of PRRs. Typically, in the resting state, the inhibitory IκB proteins interact with NF-κB p50/p65 dimers in the cytoplasm, forming an inactivation complex. Upon activation of this signaling pathway,

the inhibitory IκB proteins can be phosphorylated and degraded in a ubiquitin-proteasome manner to allow the NF-κB p65/p50 dimers to translocate to the nucleus and promote proinflammatory cytokine transcription [176].

Several studies support a critical role for Ca^{2+} signaling in regulating NF-κB activation to promote gene transcription, cytokine secretion, inflammatory responses, and tumor microenvironment remodeling in various cell and animal models (Fig. 8) [177-180]. In most cases, an increase in Ca^{2+} activates NF-κB through several different mediators. Ca^{2+} signaling can activate PKC-α to inhibit IκB-α to increase NF-κB activity and activate calcineurin to inhibit IκB-β to activate NF-κB [181]. Ca^{2+} can also activate CaMKII which can directly interact with IKK-β to modulate NF-κB signaling [182]. The Ca^{2+}/calmodulin-dependent kinase PNCK can phosphorylate the inhibitor IκB-α to activate NF-κB. In addition, several signaling pathways including ERK1/2, PI-3K, and P38, are sensitive to changes in Ca^{2+} and result in activation of NF-κB [178, 183, 184]. Several other Ca^{2+} channels and sensors also have been identified for regulating Ca^{2+} signaling in a way that affects NF-κB activation including TRPC3, TRPC6, RyR2, ORAI1, and STIM1 [179, 185-187]. Even though these Ca^{2+} channels and sensors have been identified for these regulatory roles, the detailed mechanisms for how changes in Ca^{2+} signaling result in NF-κB activation have yet to be elucidated.

Calcium Flux and Inflammasome Activation.

Ca^{2+} flux or mobilization are considered an important upstream signal in NLRP3 inflammasome activation [188, 189]. Given that the cytoplasmic concentration of Ca^{2+} is

low, the two primary sources that cause Ca^{2+} mobilization come from the opening of

plasma membrane channels and the release of intracellular ER-linked Ca^{2+} stores to

create Ca^{2+} flux in the cytoplasm.

Figure 8. Crosstalk between NF-κB and Ca²⁺ Signaling. JAK2 activates PLC-β to produce IP3 which activates IP3R to facilitate Ca²⁺ release from the ER. JAK3 also regulates Ca²⁺ release from the ER via IP3 to activate IP3R. JAK3 enhances Ca²⁺ release to the extracellular space through NCX. Ca²⁺ signaling also promotes the activation of JAK1 and STAT1 and enhances the interaction of PYK2 and JAK2 to facilitate JAK2 activation. Ca²⁺ signaling mediated by IP3R, TRPC1, and TRPC7 induce STAT3 activation. NF-κB promotes Ca²⁺ signaling through TRPC1, TRPC6, ORAI1, and STIM1, and Ca²⁺ release to the extracellular space via NCX1. Ca²⁺ signaling regulates NF-κB pathways via PKCα and PNCK, which inhibits IκB-α to enhance NF-κB, and activates calcineurin and enhances the interaction of CaMKII with IKK-β to then activate NF-κB. Ca²⁺ signaling sensitizes PI3-K, ERK1/2, and P38 signaling to activate NF-κB. The Ca²⁺ channels, including TRPC6, TRPC3, RyR2, ORAI1, and ER-resident Ca²⁺ sensor STIM1, are also involved in NF-κB activation. Reprinted from [6].

Since these pathways are linked, when one of these stores is released, the other one typically follows. An increase in cytosolic Ca^{2+} can be caused by an influx of extracellular Ca^{2+} through different Ca^{2+} channels including P2X7R, TRPM2, TRPV2, TRPA1, and TRPV1 which facilitate activation of the NLRP3 inflammasome [190-193]. RyR and IP3R are ion channels that are localized to the ER that facilitate the release of Ca^{2+} from the ER which can also sensitize the NLRP3 inflammasome [194]. Several G-protein coupled receptors (GPCR) can activate phospholipase C to produce IP3, a ligand of IP3R that has been found to promote NLRP3 inflammasome activation [195-198]. Two mechanisms have been proposed for how Ca^{2+} fluxes may induce inflammasome activation (Fig. 9). The first possibility is that Ca^{2+} promotes the interaction between NLRP3 and its adaptor ASC to activate the NLRP3 inflammasome, yet if this is the case the potential mechanisms are not well-characterized [188, 199]. Alternatively, excessive intracellular Ca^{2+} may lead to Ca^{2+} accumulation in the mitochondrial matrix through MCU. This Ca^{2+} overload leads to mitochondrial dysfunction accompanied by ROS production and release of mtDNA to the outer membrane. Ultimately, this exposed mtDNA promotes activation of the NLRP3 inflammasome [199, 200].

Complicating our understanding for a specific role of Ca^{2+} mobilization in NLRP3 inflammasome activation is the fact that K^+ efflux can act as the counter ion at the plasma membrane to regulate Ca^{2+} flux. Data for several stimuli that activate this inflammasome suggested that Ca^{2+} flux and K^+ efflux were coordinated in NLRP3

activation. For example, a classical second signal, ATP, binds to the P2X7 receptor to induce weak Ca^{2+} influx that is coupled with K^+ efflux [201].

Figure 9. Crosstalk between NLRs and Ca^{2+} Signaling. Cytosolic Ca^{2+} levels are increased by ion channels including P2X7R, TRPM2, TRPV2, TRPA1, TRPV1, and from the lysosome which facilitates activation of the NLRP3 inflammasome. The Ca^{2+} channels CaSR and GPRC6A facilitate PLC activation, including IP3 production which initiates Ca^{2+} release from the ER through IP3R. MAC also activates IP3R and RyR to mediate the ion outflow from the ER to activate the NLRP3 inflammasome. The viral Ca^{2+} channels, including EMCV 2B, HRV 2B, and SARS-CoV E mediate Ca^{2+} release from the ER and Golgi, and the viral Ca^{2+} channel FMDV 2B promotes Ca^{2+} entry. Excessive Ca^{2+} in the mitochondria mediated by MCU induces ROS production and causes mtDNA release which activates the NLRP3 inflammasome. NLRP3 inflammasome activation also contributes to the increase of Ca^{2+} in the SR. SR: sarcoplasmic reticulum, EMCV: encephalomyocarditis virus, HRV: human rhinovirus, FMDV: foot-and-mouth disease virus. Reprinted from [6].

Further demonstrating how these ion fluxes are linked, K^+ efflux promotes the release of ER Ca^{2+} stores that is then followed by the opening of plasma membrane Ca^{2+} channels. Other activators of the NLRP3 inflammasome including nigericin, alum, monosodium urate crystals, and the membrane-attack complex induce Ca^{2+} flux as well as K^+ efflux [189, 194]. In contradiction of these findings, one study showed that for some stimuli, the Ca^{2+} flux actually occurred downstream of NLRP3 and caspase-1 activation suggesting it was not critical for NLRP3 inflammasome activation [202]. Overall, the exact role of Ca^{2+} flux in NLRP3 inflammasome activation is still debated. Another source of danger signals that activate the NLRP3 inflammasome are derived from mitochondria and will be described in the following sections.

Mitochondrial Homeostasis.

Mitochondria are dynamic organelles that are necessary for energy production through oxidative phosphorylation (OXPHOS), regulation of apoptosis, integration of various metabolic pathways, and maintenance of calcium homeostasis [203]. To carry out these processes, it is critical to preserve mitochondrial integrity and homeostasis which occurs through regular fission and fusion. A mitochondrion has several structural characteristics including an outer membrane, intermembrane space, inner membrane, and matrix. Of particular interest here is the mitochondrial matrix which contains the mitochondrial genome which consists of a 16.5 kb double-stranded closed circular DNA [204]. The mitochondrial genome contains 37 genes that encode 13 OXPHOS proteins, 2 ribosomal RNAs, and 22 transfer RNAs, yet most mitochondrial genes are encoded in

the nucleus and are transported to the mitochondria following synthesis in the cytoplasm.

Mitochondrial Fission and Fusion.

Mitochondrial dynamics are mediated by fission and fusion which results in a constant change in the number and morphology of mitochondria. These processes ensure that there is a sizable group of mitochondria that have optimal OXPHOS activity by moving and distributing mitochondrial content [205]. Mitochondrial fusion uses three membrane GTPases, mitofusin 1 (MFN1), mitofusin 2 (MFN2), and optic atrophy protein 1 (OPA1), to join two mitochondria at the outer and inner membrane interfaces. Conditions that promote fusion include inhibition of the mammalian target of rapamycin (mTOR)-induced autophagy, protein synthesis, and starvation [206-208]. Thus, mitochondrial fusion is necessary to promote optimal mitochondrial function because it allows for the exchange of gene products and metabolites.

MFN1 and MFN2 are located on the outer mitochondrial membrane and promote fusion. Interestingly, MFN2 is also located on the ER membrane where it connects the ER to mitochondria which allows for calcium uptake by the mitochondria [209]. Perhaps some similarities exist in the state of the cell between MFN2 KO and VMP1 KO [5]. MFN1 and MFN2 facilitate docking of the mitochondria at the outer membranes and their subsequent fusion. Under conditions of cellular stress, JNK phosphorylates MFN2 which recruits an E3 ubiquitin ligase to ubiquitinate MFN2 for proteasomal degradation. Degradation of MFN2 causes mitochondrial fragmentation and enhanced apoptotic death [210]. Another mechanism that regulates mitochondrial turnover is mediated by

MFN2 which recruits Parkin to damaged mitochondria and ultimately results in their degradation by mitophagy [211].

The other quality control mechanism for mitochondria is fission which is the process by which a mitochondrion divides into two mitochondria. Some of the reasons for fission include inheritance and partitioning of organelles during cell division, cytochrome C release during apoptosis, and to ensure the proper distribution of mitochondria [212-214]. Fission also promotes the removal of damaged mitochondria by mitophagy. When fission is not functioning properly, there is unbalanced fusion that results in an increase in the number of elongated mitochondria [205]. Alternatively, disruption in fusion leads to more fragmented mitochondria [215]. Fission is primarily coordinated by a large dynamin-like GTPase, known as dynamin-related protein 1 (DRP1). Mitochondrial division occurs where the ER interacts with the mitochondria. At this interaction point, the mitochondria are constricted then DRP1 is recruited. The average diameter decreases from around 300-500 nm to around 150 nm [205]. Ca^{2+} plays a role here where constriction of the inner mitochondrial membrane (IMM) at the mitochondria-ER contact site is Ca^{2+}-dependent [216]. There are many post-translational modifications of DRP1 that regulate the activation of fission [204]. Fission and fusion are two cellular processes that aim to maintain mitochondrial homeostasis and proper function in the cell.

Mitochondria-derived Danger Signals and Inflammasome Activation.

Another key upstream event of NLRP3 activation is mitochondrial dysfunction and the release of mitochondrial ROS (mtROS) and mtDNA into the cytosol (Fig. 10).
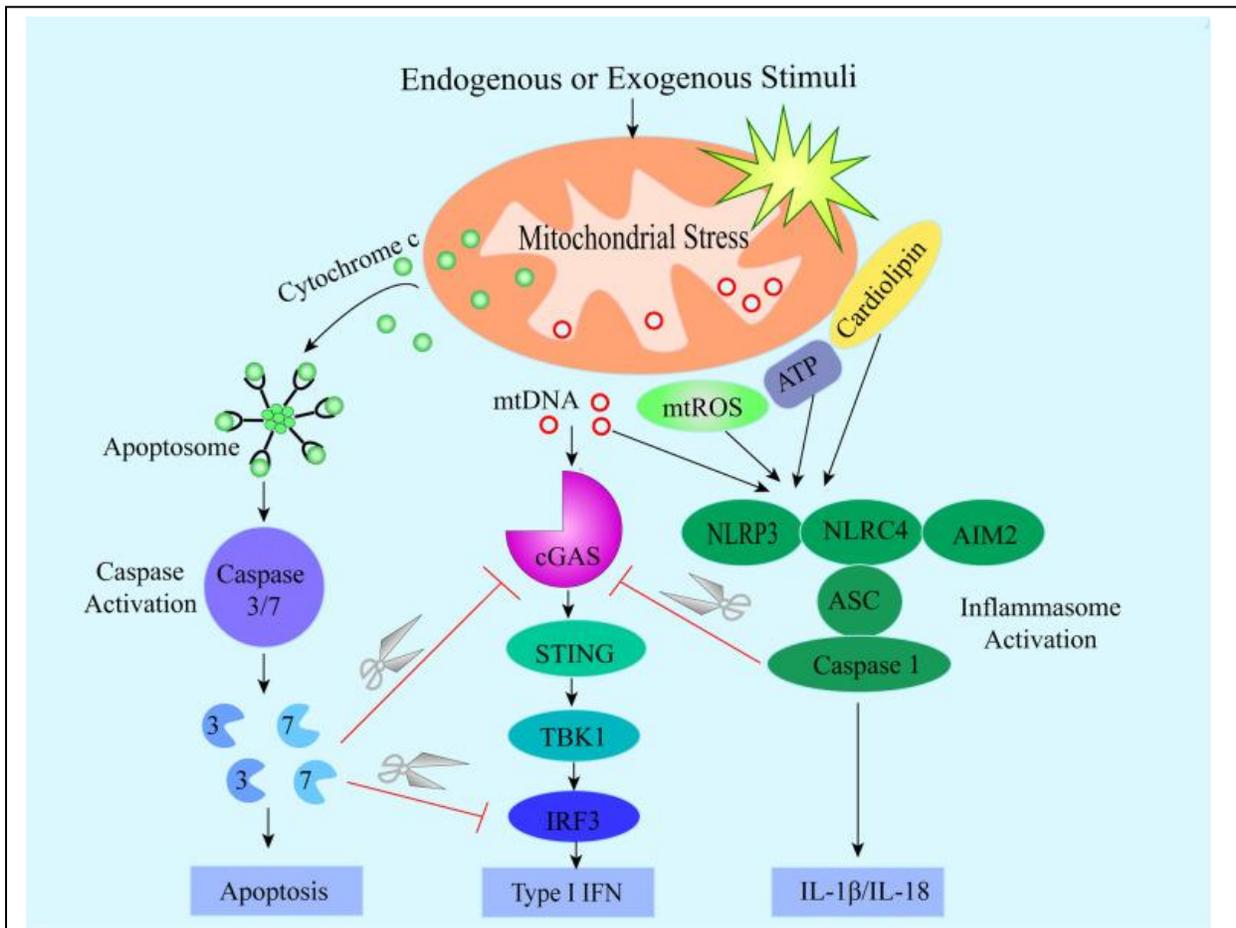
Figure 10. Mitochondrial Stress Can Trigger Innate Immune Responses.
Endogenous or exogenous stimuli induce cell stress that either directly or indirectly cause mitochondrial stress. Mitochondrial stress results in the release of mtROS, mtDNA, and ATP into the cytosol and extracellularly, cardiolipin externalization and cytochrome c release into the cytosol. Cytosolic mtDNA activates the cGAS-STING pathway to produce type I interferon. Signals including mtROS, mtDNA, cardiolipin, and ATP engage in the activation of several inflammasomes that leads to IL-1β and IL-18 processing and secretion. Cytosolic cytochrome c triggers activation of apoptotic caspase-3/-7 which inhibits type I IFN production via cleaving cGAS and IRF3. Similarly, caspase-1 can cleave cGAS to prevent type I IFN expression. Reprinted from [4].

Under normal conditions, mitochondria produce reactive oxygen species (ROS) as a by-product of oxidative phosphorylation, but when the cell is under stress, mtROS levels significantly increase. Thus, an important regulator of NLRP3 activation is mitophagy, a

cellular process that removes damaged and dysfunctional mitochondria and reduces mtROS production [217]. A previous study showed that a novel pathway involving DRP1-Parkin1-VMP1 is needed for the selective degradation of damaged mitochondria through mitophagy in acute pancreatitis [150]. When mitophagy is impaired as is the case in VMP1 KO cells, there may be decreased negative regulation of the NLRP3 inflammasome and an accumulation of damaged mitochondria.

Another important signal in NLRP3 activation is circulating mtDNA released from damaged cells which acts as a DAMP [218]. mtDNA release from the cell has been found to be dependent on GSDMD pores in BMDMs activated by LPS and nigericin [219]. Endogenous newly synthesized mtDNA also has been considered a critical component of the NLRP3 signaling pathway [220-222]. Under conditions of oxidative stress, mtROS and Ca^{2+} together open the mPTP resulting in the release of mtDNA into the cytoplasm [222, 223]. Several activators of the NLRP3 inflammasome induce the rapid release of oxidized mtDNA into the cytoplasm [221]. Data showed that oxidized mtDNA activates the NLRP3 inflammasome, and non-oxidized mtDNA preferentially activates the AIM2 inflammasome [221]. In contradiction to previous findings, one study utilized a genetic deletion approach and found that there was no role for mPTP or mitophagy in NLRP3 activation [224]. Again, like with Ca^{2+} flux, there is conflicting data about the role for mitochondria in NLRP3 activation, and further studies are needed to elucidate the essential signals for NLRP3 inflammasome activation.

In addition to the role of mitochondria in NLRP3 activation, it is becoming increasingly evident that mitochondria are the docking sites for inflammasome

assembly. Typically, NLRP3 is a cytoplasmic protein that associates with the ER in unstimulated cells, but upon activation, NLRP3 becomes associated with mitochondria and the mitochondria-associated membrane (MAM) [217, 225]. While several mitochondrial proteins have been connected to inflammasome activation, the most relevant one in the context of this study is cardiolipin. Cardiolipin, a mitochondrial phospholipid of the inner membrane, is exposed on the outer membrane upon mitochondrial stress where it acts as the binding site for proteins associated with autophagy and apoptosis [226]. Cardiolipin has been shown to be necessary for inflammasome activation and binds NLRP3 and full-length caspase-1 independently [227, 228]. NLRP3 stimuli have been found to promote trans-Golgi network disassembly into vesicles which recruits NLRP3 and promotes NLRP3 aggregation needed for downstream ASC oligomerization and caspase-1 activation [229]. It is thought that perhaps then K^+ efflux dependent and mitochondria dependent NLRP3 activation may be separate pathways that converge on trans-Golgi disassembly, yet a unifying model for the various signals that activate the NLRP3 inflammasome has remained elusive.

cGAS-STING and Inflammasome Activation.

There are multiple cytosolic DNA sensors including cyclic GMP-AMP synthase (cGAS) which are involved in DNA-dependent immune responses. After cGAS recognizes cytosolic DNA, cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) is produced. Next, cGAMP binds to the stimulator of interferon genes (STING) which is localized on the ER. Once activated, STING translocates to the Golgi apparatus, where it interacts with TBK1 to then trigger IRF3

activation and translocation to the nucleus to induce transcription of interferon genes. Additionally, STING recruits IκB kinase (IKK) to phosphorylate IκBα which results in activation of NF-κB and induction of proinflammatory cytokine gene expression [230]. Previously, it had been thought that sensing of cytosolic DNA resulted in activation of the cGAS-STING pathway to trigger an antiviral response by inducing type I interferons [231, 232], whereas sensing of cytosolic DNA by AIM2 induced a proinflammatory response specifically through inflammasome activation [233, 234]. Yet the work of Gaidt et al. supported the idea that in human myeloid cells, AIM2 was not required for inflammasome activation. Instead, in human cells, activation of the cGAS-STING pathway led to NLRP3 inflammasome activation [235]. Furthermore, a recent study identified that mtDNA was released through the mPTP to activate the cGAS/STING pathway in amyotrophic lateral sclerosis [236]. Overall, it appears that mtDNA released from dysfunctional mitochondria can be sensed by the cGAS/STING pathway to activate the NLRP3 inflammasome.

Autophagy.

Macroautophagy or autophagy as it will be referred to hereafter is a cellular process that facilitates the degradation of unnecessary or dysfunctional cellular components (Fig. 2). The first step of autophagosome formation starts with the initiation and nucleation of a crescent-shaped IM that ultimately expands and seals [28]. Autophagosome formation occurs at the ER [237, 238]. When autophagy is induced, autophagosome formation occurs at PI(3)P-enriched ER subdomains called

omegasomes [237]. ER-mitochondrion contact sites, regions called the mitochondria-associated ER membrane are also involved in autophagosomal biogenesis [238, 239]. Three-dimensional (3D) electron tomography showed that the IM was located between ER cisternae during expansion of the autophagosome [240, 241]. It was thought that these contacts were important for the transport of lipids from the ER to allow for expansion of the IM although other sources were thought to also contribute lipids [29, 242]. When the IM closes, the outer surface of the autophagosome dissociates from the ER [240]. The autophagosome fused with endosomes to undergo a maturation process that ultimately resulted in it fusing with lysosomes [242]. As mentioned before, autophagy is impaired in VMP1 KO cells at the stage of autophagosome biogenesis and at the stage of autophagosome/lysosome fusion.

Ca^{2+} Modulation of IM Association with the ER.

IM association with the ER can be modulated by changes in Ca^{2+} levels. Interestingly, treatment with thapsigargin, a specific inhibitor of SERCA, which perturbs intracellular calcium ion homeostasis and induces ER stress, prevents detachment of IM/autophagosomes from the ER [243, 244]. SERCA helps to maintain the cytosolic [Ca^{2+}] at low resting levels by transporting Ca^{2+} from the cytosol into the ER lumen [245]. In cells treated with thapsigargin, the IMs expanded into spherical-shaped structures that do not completely seal and remain associated with the ER [244]. Yet use of a low concentration of thapsigargin (100 nm) that does not elicit sustained or detectable changes in cytosolic Ca^{2+} levels was still able to block autophagy [244]. Collectively, these data suggested that VMP1 KO cells have persistent contact between

the ER and various organelles due to the ability of VMP1 to regulate SERCA activity and local $[Ca^{2+}]$ [5, 244].

Ca^{2+} Homeostasis and Mitochondrial Membrane Potential.

Calcium crosstalk occurs in the region between the ER and mitochondria known as the mitochondria-associated membrane to control cellular homeostasis [246]. Cellular Ca^{2+} levels are maintained through Ca^{2+} influx through cation channels located on the plasma membrane and the release of Ca^{2+} from intracellular stores. The main intracellular source of Ca^{2+} is the ER. Mitochondria are the main sources of ATP production in the cell, and there is an important link between mitochondrial levels of calcium and mitochondrial dynamics and function. Mitochondria can regulate the amplitude and timing of intracellular Ca^{2+} signals through mitochondrial calcium buffering capacity and mitochondrial functional interactions with other channels or organelles. Mitochondria can buffer Ca^{2+} levels between 50 and 500 nM in several cell types [247-249]. Mitochondria are particularly important in buffering severe Ca^{2+} overloads which tend to be associated with pathological conditions [249]. Close contact between mitochondria and cellular Ca^{2+} gates located in the ER and the cell membrane creates microdomains where an increase of $[Ca^{2+}]_{mito}$ parallels cytosolic Ca^{2+} signals [250]. Contacts between mitochondria and the ER increases $[Ca^{2+}]_{mito}$ through the opening of the IP_3-gated channels in the ER. At the sites of these contacts, the mitochondrial surface is exposed to higher concentrations of Ca^{2+} than at other sites exposed to the bulk cytosol [251, 252]. Mitochondrial Ca^{2+} overload results in mitochondrial fragmentation [253].

When the cell is undergoing increased activity, transient cytoplasmic Ca^{2+} increases are sequestered by mitochondria through the mitochondrial Ca^{2+} uniporter which results in increased matrix Ca^{2+} levels [254-258]. Ca^{2+} enters mitochondria through the electrochemical gradient created by the mitochondrial membrane potential, and it exits the mitochondria via the Na^+-Ca^{2+} and the H^+-Ca^{2+} exchangers (NCX and HCX) in non-excitable cells [259]. In the mid-20th century, it was observed that high levels of Ca^{2+} result in mitochondrial swelling and dysfunction [260, 261]. These findings resulted in the proposal of the existence of the mitochondrial permeability transition pore (mPTP). The mPTP is a non-specific channel located in the inner mitochondrial membrane. mPTP is engaged for prolonged periods of time in response to elevated intracellular Ca^{2+} levels and/or oxidative stress ultimately resulting in mitochondrial rupture. Opening of MPTP acutely results in the loss of mitochondrial inner membrane potential and release of molecules up to 1500 Da but long-term opening results in organelle swelling and rupture that leads to mitochondrial dysfunction and cell death [262, 263]. Typically, mitophagy is a specialized process that removes dysfunctional mitochondria. Upon mitochondrial depolarization, phosphatase and tensin homolog deleted on chromosome 10-induced kinase 1 (PINK1) and beclin 1 (BECN1) relocalize to the surface of damaged mitochondria at the MAM site. These proteins then recruit others to result in autophagosome formation and mitophagy of damaged mitochondria [264]. Therefore, elevated Ca^{2+} levels can cause mitochondria to lose their membrane potential. In cells with dysregulated autophagic processes, there will be an accumulation of damaged mitochondria such as is the case in VMP1 KO cells.

VMP1 and Mitophagy.

The presence of mitochondrial stress can activate several innate immune pathways including NLRP3 inflammasome activation and apoptosis [4]. To control mitochondrial stress responses, a mitochondrial quality control mechanism that is a selective type of autophagy termed mitophagy exists to selectively degrade damaged mitochondria [4]. When mitophagy is active, mitochondria are engulfed in double-membrane vesicles that then fuse with lysosomes for degradation. When mitochondria lose their membrane potential, a sign of mitochondrial dysfunction, PTEN-induced kinase 1 (PINK1) accumulates in the mitochondrial outer membrane. Parkin1 then ubiquitinates these damaged mitochondria for autophagic degradation [265-267]. Prior work has suggested that VMP1 was required for mitophagy. In an acute pancreatitis model, mitochondria were a direct target of damage [150]. Data showed that VMP1 was involved and required for mitophagy during acute pancreatitis and in the case of decreased expression of VMP1 there was reduced mitochondrial degradation [150]. In the pancreatitis model, there is a redistribution of VMP1 around rounded mitochondria [150]. Overall, these data suggested that in models with decreased expression of VMP1 there would be an accumulation of damaged mitochondria resulting from decreased mitophagy.

Secretory Autophagy.

Secretory autophagy is a cellular process that facilitates the unconventional secretion of cytosolic cargo. The most often thought of cargo are leaderless cytosolic proteins which lack the N-terminal signal that normally allows for conventional protein

secretion through the endoplasmic reticulum and Golgi apparatus. Other types of cargo for unconventional secretion include cytosolic proteins with extracellular functions, aggregate forming proteins, cytoplasmic organellar material, and microbial release from cells for transmission. In the case of secretory autophagy, double membrane autophagosomes form and engulf cytoplasmic components except instead of fusing with lysosomes for degradation of their contents they fuse with the plasma membrane to expel their contents from the cell [268]. One important example of a cytosolic protein that undergoes unconventional secretion is IL-1β [269-271]. A recent study found that in cells that have a defect in mitophagy as is the case with VMP1 KO cells, mitochondria can still be cleared from cells in a process independent of lysosomal degradation. Mitochondria can instead be cleared by extracellular release through a secretory autophagy pathway that was defined as Autophagic Secretion of Mitochondria (ASM). It was observed that when there was increased ASM, there was more activation of the cGAS/STING pathway in recipient cells [272]. Clearly, cells with impaired autophagic processes can utilize secretory autophagy to remove unwanted cellular contents that cannot be degraded through autophagy.

Another way that cargo can be released from cells is through extracellular vesicles (EVs). EVs are a heterogenous population of membrane-enclosed cargoes that are released from cells to expel "waste" or to mediate intercellular signaling. Using proteomics approaches, several studies have found that various inflammasome activators including calcium oxalate, monosodium urate crystals, ATP, β-glucans, and viral RNA among others promote the release of EVs [273-278]. ATP activates the

NLRP3 inflammasome by binding to the P2X7R which allows for K^+ efflux and the rapid release of EVs [273]. Several studies support the idea that gal-3 is released in EVs specifically through recruitment to multivesicular bodies by TSG101 [279-282]. Overall, under inflammatory conditions, cytoplasmic cargo can be released from cells through several different mechanisms including secretory autophagy and GSDMD pores.

Concluding Remarks.

Macrophages have intrinsic pathways to modulate inflammatory signaling to promote homeostasis. However, it is likely that in macrophages lacking VMP1 there is aberrant dysregulation to an extent that cannot be compensated for by these pathways. An overview for how Ca^{2+} flux and mitochondria-derived danger signals activate the NLRP3 inflammasome has been provided in the previous sections. Given what is known about the function of VMP1, it is feasible that VMP1 can regulate inflammatory responses by modulating SERCA activity and mitophagy. As mentioned before, VMP1 is localized to the ER where it interacts with SERCA, a Ca^{2+}-ATPase, to modulate the local Ca^{2+} levels in the cytoplasm [5]. Previous work demonstrated that increased cytoplasmic $[Ca^{2+}]$ promoted the assembly of inflammasome components, but how exactly Ca^{2+} mediates this response is not well understood [188]. In activated cells or cells with dysregulation, there was an increase in the presence of DAMPs such as mtDNA which are known to activate the inflammasome [217, 220, 283]. Prior work demonstrated that VMP1 expression was required for mitophagy, the process that specifically degrades damaged mitochondria which are a source of DAMPs [150]. While it has been shown that VMP1 can modulate local $[Ca^{2+}]$ and mitochondrial homeostasis,

it had yet to be determined whether or how VMP1 might regulate inflammatory responses until now.

Previous studies have shown that VMP1 relocalizes to rounded, damaged mitochondria in pancreatitis [150]. When VMP1-localized autophagosomes were isolated, they contained damaged mitochondria suggesting that VMP1 has a role in maintaining mitochondrial homeostasis [150]. Additionally, in cells with downregulated VMP1 expression, there was reduced mitochondrial degradation [150], and in HeLa cells and Cos-7 cells with depleted VMP1, altered mitochondrial morphology, size and increased or absent cristae were observed [15]. Furthermore, our preliminary data and other work suggested that there was an accumulation of SQSTM1/p62 in VMP1 deficient cells which is typically evidence of impaired autophagy [284]. p62 is an autophagy substrate protein that acts as a receptor protein in signaling pathways that include NFκB, Nrf2, and mTOR which are either directly part of inflammatory pathways or regulate inflammatory responses [285]. Therefore, we hypothesize that VMP1 may also regulate inflammatory responses by promoting mitochondrial homeostasis and that in KO cells potential accumulation of damaged mitochondria will prime cells to exacerbate their responses to additional inflammatory stimuli. The goal of this dissertation was to characterize a novel regulator of inflammatory responses, VMP1, such that we gain new insight into how dysregulated host cellular responses affect inflammatory responses to pathogen and damage associated signals.

CHAPTER TWO

MATERIALS AND METHODS

Cell Culture, Differentiation, and Treatments.

HEK293T and THP-1 cells were obtained from the American Type Culture

Collection (ATCC). Cells were cultured with 5% CO_2 at 37°C in either DMEM or RPMI

supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco),10 μg/mL

ciprofloxacin hydrochloride, 100 IU/mL penicillin, and 100 μg/mL streptomycin. THP-1s

were differentiated by adding phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich,

P1585) at a concentration of 1 μg/mL for 48 h. The cells were then allowed to rest for 72

h prior to treatment. Unless otherwise noted, the cells were treated with 100 ng/mL

lipopolysaccharide from *Escherichia coli* O55:B5 (Sigma-Aldrich, L4524) or were

transfected with 50 μg/mL poly(I:C) (Sigma-Aldrich, P9582) using Lipofectamine 2000

(Fischer Scientific, 11-668-019) following the manufacturer's protocol for 4 h. The media

was changed and then some wells were treated with 5 mM adenosine 5-triphosphate

disodium salt hydrate (ATP) (Sigma-Aldrich, A2383) for 30 min. To measure the

response to α-syn, α-syn fibrils were obtained as described previously, and 1 μM α-syn

was added to differentiated THP-1s for 24 h [286]. To inhibit NLRP3 inflammasome

activation, 10 μM MCC950 (Invivogen, inh-mcc) or DMSO control were added to the

differentiated THP-1s in serum-free RPMI for 1 h prior to the addition of ATP. To test

SERCA inhibition, 1 μm thapsigargin (Tocris, 1138) was added at the same time as any

inflammatory stimuli including when it served as a control. The supernatant or cells were then collected for analysis. To deplete extracellular Ca^{2+}, cells were pretreated with 2.2 mM EGTA (EMD Millipore Corp, 324626) for 30 min. To release Ca^{2+} stores, then either 1 µM thapsigargin, 1 µM ionomycin (Sigma-Aldrich, I9657) or 2.2 mM EGTA was added to cells for 4.5 h. The supernatant or cells were then collected for further analysis.

Cloning and Generation of Stable Cell Lines.

GSDMD knockout THP-1 cell lines were generated using a modified version of the LentiCRISPRv2 plasmid (Addgene plasmid number 52961, a gift from Feng Zhang) that has the puromycin resistance cassette replaced with a blasticidin resistance cassette to create LentiCRISPRv2-blasticidin [287]. The following oligonucleotide guide RNA sequence was annealed and cloned into LentiCRISPRv2-blasticidin: GSDMD guide RNA 5'-CCACGTACACGTTGTCCCCG-3' as described previously. Cells transduced with the LentiCRISPRv2-blasticidin backbone served as a control for selection. VMP1 knockout THP-1 cell lines were generated using a modified version of the LentiCRISPRv2 plasmid that has the puromycin resistance cassette replaced with a G418 resistance cassette to create LentiCRISPRv2-G418 [287]. The following oligonucleotide guide RNA sequence was annealed and cloned into LentiCRISPRv2-G418: VMP1 guide RNA 5'-CTTTTGTATGCCTACTGGAT-3' as described previously [12]. Cells transduced with the LentiCRISPRv2-G418 backbone served as a control for selection. To generate stable cell lines, lentivirus was prepared by transfecting equal amounts of VSV-G, psPAX2 (from Didier Trono, NIH AIDS Reagent program [catalog number 11348]) [288, 289], and LentiCRISPRv2-G418 (either the backbone or the clone

containing the guide RNA of interest) using polyethyleneimine (PEI) into HEK293T cells.

Retrovirus was prepared by transfecting equal amounts of VSV-G, pCigB, and

pMSCVpuro-Mito-Pericam using PEI into HEK293T cells. Viral supernatant was

harvested 48 h post-transfection and filtered through 0.45-µm filters (Millipore). The

concentrated supernatant was applied to THP-1 cells by spinoculation at 13°C for 2 h at

1,200 x *g*. Media was changed 24 h later. Forty-eight hours after transduction, geneticin

(G418) (Gibco) was added to the cells at a concentration of 0.5 mg/mL. Following 3-4

weeks of selection, lymphocyte separation media was used to remove dead cells, and

healthy cells were collected to validate the knockout by western blot.

To measure caspase-1 activation, THP-1s were transduced with lentiviral vector

prepared as described above with a caspase-1 biosensor containing the IQAD amino

acid target sequence as described previously [290].

Sandwich ELISAs.

Cell culture supernatants were analyzed using the following kits: Human IL-1

beta/IL-1F2 DuoSet ELISA (R&D Systems, DY201-05) for IL-1β, Human IL-6 DuoSet

ELISA (R&D Systems, DY206-05) for IL-6, and Human Galectin-3 DuoSet ELISA for

gal-3 (R&D Systems, DY1154). The manufacturers' protocols were followed.

Alternatively, gal-3 protein levels were also measured using an in-house sandwich

ELISA described previously [291]. Briefly, mouse anti-LGALS3 B2C10 (Santa Cruz

Biotechnology, SC-32790) was diluted in pH 9.6 carbonate buffer to a final

concentration of 1 µg/mL to coat a 96-well Maxisorp ELISA plate (Nunc, 44-2402-22) at

4°C overnight on an orbital shaker. Between each step the wells were washed 3x-5x

with PBS containing Tween-20. The wells were blocked 1:1 with RPMI supplemented

with 10% characterized FBS and PBS for 2 h at RT. The culture supernatant was then

added to the wells. The standard curve was generated by serially diluting recombinant

gal-3 (Abcam, ab89487). Biotin conjugated rat anti-LGALS3 (M3/38; Millipore Sigma,

125402) was diluted to a final concentration of 500 ng/mL in PBS with 1% bovine serum

albumin (BSA) (Sigma-Aldrich, A7906) and added to the wells for 2 h on a rocker at RT.

Then streptavidin HRP (ImmunoReagents, Ba-103-HRPX) was diluted to 1 µg/mL and

incubated for 30 min at RT on a rocker. The HRP signal was detected with the addition

of 1 x 3,3',5,5-tetramethylbenzidine (Invitrogen, 00-4201-56) and then the reaction was

quenched with 2 N sulfuric acid. The absorbance was read at 450 nm on a PowerWave

XS plate reader (BioTek Instruments) with Gen5 software. The standard curve was fit

with a 4-Parameter Logistic (4PL) curve.

Quantitative Real-Time PCR.

Cells were treated as described above and RT-PCR was used to measure

changes in gene expression. Total RNA was purified from cell lysates following

treatment using the NucleoSpin RNA Plus extraction kit (Macherey-Nagel, 740984.250).

cDNA was synthesized using the GoScript Reverse Transcriptase System (Promega,

A5004). Quantitative PCR was performed using gene specific primers and Itaq™

Universal SYBR (Bio-Rad, 1725124). The following primer sets were used in this study:

VMP1 fwd, 5'-GTGGCTTTCATTGGTGCTGTCC-3'; VMP1 rev, 5'-

GAGTTCAACCGCTGCTGGATTC-3'; proIL-1β fwd, 5'

AATCTGTACCTGTCCTGCGTGTT-3'; proIL-1β rev, 5'-

TGGGTAATTTTTGGGATCTACACTCT-3'; IL-6 fwd, 5'-GGAGACTTGCCTGGTGAAAA-

3'; IL-6 rev, 5'-ATCTGAGGTGCCCATGCTAC-3'; lgals3 fwd, 5'-

GCCAACGAGCGGAAAATGG

-3'; lgals3 rev, 5'-TCCTTGAGGGTTTGGGTTTCC-3'; GAPDH fwd, 5'-

GCACCGTCAAGGCTGAGAAC-3'; GAPDH rev, 5'-GCCTTCTCCATGGTGGTGAA-3'.

GAPDH was utilized as a housekeeping gene for normalization.

RNA-Sequencing and Pathway Analysis.

Control or VMP1 KO differentiated THP-1s were left untreated or treated with 100

ng/mL LPS (4 h) then 5 mM ATP (30 min). Supernatant was collected and analyzed by

gal-3 ELISA as described above to ensure that the sample phenotype was consistent

with previous experiments. RNA was isolated using the NucleoSpin RNA Plus extraction

kit. Part of the RNA was saved for qPCR to assess proIL-1β, IL-6, and gal-3 gene

expression as described above. RNA samples were submitted to the University of

Chicago Genomics Facility to assess the concentration and quality of the RNA.

Samples that passed the check were then used for library preparation and whole

genome sequencing using Illumina NovaSeq. Pathway analysis was performed

following a previously published protocol [292]. Heatmaps were generated using the

pheatmap function (RRID:SCR_016418) in R.

Western Blotting.

Protein was isolated by lysing pelleted cells using lysis buffer containing 100 mM

Tris, pH 8.0, 1% NP-40 (Thermo Fischer Scientific, 85124), 150 mM sodium chloride

and Pierce protease inhibitor cocktail (Thermo Fischer Scientific, 32953) on ice for 30

min. The lysates were centrifuged at 4°C for 10 min at 10,000 x *g* and then the supernatant was collected and transferred to a new tube. Protein from the supernatant was concentrated using ethanol precipitation. Prior to collecting supernatant samples, for the media change before the addition of ATP for 30 min, the media was replaced with serum free media. One volume of protein solution was combined with 9 volumes of 100% cold ethanol. The samples were vortexed and stored at -20°C overnight. The samples were then centrifuged for 10 min at 15,000 x *g.* The supernatant was removed, and the pellet was washed with cold ethanol. The sample was vortexed and repelleted for 10 min at 15,000 x *g.* The ethanol was aspirated off. The samples were dried to eliminate ethanol residue, and then the samples were resuspended in 6x SDS. The protein concentrations were determined by Pierce 660 nm protein assay (Thermo Fischer Scientific, 22660). In brief, 2x SDS was added to the lysed sample and boiled at 95°C for 5 min. An equal amount of protein was loaded into a 12% polyacrylamide gel or a precast 4-20% gradient polyacrylamide gel (Mini-PROTEAN TGX Precast Polyacrylamide Gels, Bio-Rad, 4561096) for SDS-PAGE. After separation, the proteins were transferred to a nitrocellulose membrane (Bio-Rad, 162-0115) and probed overnight at 4°C unless otherwise indicated with the primary antibody diluted in powdered milk block solution at 2.5 g/50 mL of Tris-buffered saline, 0.1% Tween 20 (Sigma-Aldrich, P7949-500ML). The primary antibodies were rabbit anti-GSDMDC1 (1:1000; Novus Biologicals, NBP2-33422), mouse anti-galectin-3 clone Gal397 (1:1000; BioLegend, 126701), rabbit anti-VMP1 (1:1000; StressMarq Biosciences, SPC-680D), mouse anti-β-actin (1:1000 at RT for 1 hr; Proteintech, 66009-1-Ig), rabbit anti-caspase-

3 (1:1000; Cell Signaling, 9662S), rabbit anti-cleaved caspase-3 (Asp175) (1:1000; Cell Signaling, 9661S), rabbit anti-LC3B (1:1000; Sigma-Aldrich, L7543), and rabbit anti-p62 (1:500; Cell Signaling, 7695S).

The nitrocellulose membrane was washed in Tri–buffered saline, 0.1% Tween 20 and probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse (Thermo Fischer Scientific, 12-349) or HRP-conjugated anti-rabbit secondary antibody (Thermo Fisher Scientific, 12-348) diluted in milk block solution at 1:10000 for 30 min. HRP was detected with the addition of SuperSignal West Fempto Chemiluminescent Substrate (Thermo Fischer Scientific, PI34096). Chemiluminescence levels were measured using the FluorchemE Imaging System (Protein Simple).

Caspase-1 Activation Assays.

For the FAM-FLICA caspase-1 activation assay, following treatment, the cells were incubated with FAM-FLICA caspase-1 (YVAD) substrate following the manufacturer's protocol (Immunochemistry Technologies, 97). Briefly, the FLICA substrate that was resuspended in DMSO was diluted in PBS 1:5. The diluted substrate was added to the wells at a final concentration of 1:30. The plate was incubated at 37°C for 1 h. The cells were washed with apoptosis buffer from the kit and were allowed to sit for 10 min in the incubator. Hoechst dye was added at a dilution of 0.5% to apoptosis buffer. The cells were incubated with the dye for 15 min then the cells were washed once with 1x apoptosis buffer then fresh buffer was added prior to imaging. A 20x lens was used to take 10 images per well. Data were collected by z-stack imaging and were analyzed as maximum intensity projections (MIPs). Cells were imaged using z-stacks

with 1 μm between each stack and a total of 5 z-stacks. A surface algorithm was built in Imaris around each individual cell, and the data were displayed as the intensity max of each individual cell for a given treatment.

To measure caspase-1 activation using the biosensor, following treatment, cells were lysed with 1x passive lysis buffer (Promega, E1941). Lysates were transferred to a white 96 well plate in duplicate or triplicate. Firefly luciferase substrate was added, and luminescence (relative light units) was quantified.

Lactate Dehydrogenase Assay.

Lactate dehydrogenase (LDH) release was measured using a previously published protocol [291, 293]. To measure LDH release, supernatant was collected 3 h after signal 2. The samples were plated in duplicate in a clear 96-well plate. Signal was measured by reading the absorbance at $\lambda = 490$ nm on a Synergy HTX Multi-Mode plate reader (BioTek Instruments) with Gen5 software.

Purification of Extracellular Vesicles.

Either control or GSDMD KO THP-1s were differentiated as described above in 10 cm dishes at a density of 10,000,000 cells per dish. Cells were treated as described above. Then the supernatant was collected to purify the EVs. EVs were isolated in a 15 mL conical which was centrifuged in a tabletop centrifuge at 300 x *g* for 20 min at 4°C. The supernatant was collected and added to Beckman Coulter polycarbonate centrifuge tubes (#344059). The samples were spun at 10,000 x *g* with the SW41 TI Beckman rotor in an Optima L-90K ultracentrifuge at 4°C for 30 min. The supernatant was then transferred to a new centrifuge tube and spun at 100,000 x *g* for 90 min at 4°C. The

supernatant was discarded, and the centrifuge tube was filled with PBS prior to a second spin at 100,000 x g for 90 min at 4°C. The pellet was then resuspended in 100 µL PBS.

Extracellular Vesicle Immunofluorescence Staining and Imaging.

To adhere EVs to coverslips, 30 µL of resuspended EVs were added to 470 µL PBS then was added into a 24-well plate containing a glass coverslip (Fischerbrand Cover Glasses, 12-545-J, 22 x 60-1). The plate was spinoculated by centrifugation at 13°C for 2 h at 1200 x g onto the coverslips. The EVs were then fixed in a solution of 0.1 M PIPES containing 3.7% formaldehyde, methanol free (Polysciences, 04018-1) for 10 min and washed 3x with PBS. The EVs were permeabilized with a 0.1% solution of saponin in block solution composed of 500 µL PBS supplemented with 10% normal donkey serum and 0.01% NaN$_3$ for 5 min. After washing the coverslips 3x in PBS, the coverslips were incubated with the primary antibodies, rat M3/38 anti-galectin-3 antibody (1:500; Biolegend, 125402), mouse anti-CD63 (1:2000; BD Pharminogen, 556019), mouse anti-CD9 (1:2000; BD Pharminogen, 555370), and mouse anti-CD81 (1:2000; BD Pharminogen, 9149590) for 1 h at RT. The coverslips were washed 3x in PBS. The coverslips were then incubated with the secondary antibodies including 488 conjugated donkey anti-mouse (1:300; Jackson ImmunoResearch Laboratories, 715-545-150) and 647 conjugated donkey anti-rat (1:300; Jackson ImmunoResearch Laboratories, 712-605-150) in block solution for 20 min at RT. The coverslips were then washed 3x with PBS. The coverslips were fixed and mounted (Electron Microscopy

Sciences, Fluoro-gel with Tris buffer, #17985-11) onto slides (Globe Scientific Inc.,

Diamond White Glass 25 x 75 x 1 mm, 0.5 gloss, #1380-30).

EVs were imaged on a DeltaVision wide-field fluorescence microscope (Applied

Precision, Inc.). It has a digital camera (CoolSNAP HQ2; Photometrics). An oil

immersion Olympus Plan Apo 60x objective lens (N.A.=1.42) was coated with 1.518

refraction index low autofluorescence immersion oil, Olympus Type F (Fischer

Scientific, NC0297589). For each coverslip, 20 images were acquired with 40 z-stacks

per image at 0.2 µm per z-stack for 8 µm total. For image analysis, the spots algorithm

in Imaris was used. The algorithm was built around the 488 tetraspanin channel. The

estimated XY diameter was 0.5 µm and the estimated Z diameter was 1 µm.

Calcium Live Cell Imaging.

To measure $[Ca^{2+}]_{cyt,}$ cells were incubated at RT with the high affinity Ca^{2+}

indicator Fluo-4/AM (ThermoFisher Scientific, F14201) for 15 min in Tyrode solution

(NaCl 135 mM; KCl 4 mM; $CaCl_2$ 3 mM; $MgCl_2$ 1 mM; glucose 10 mM; HEPES 10 mM;

pH 7.4). Fluo-4 was measured at an excitation/emission of 488/>515 nm. Cells were

perfused with Tyrode solution for 2 min for a baseline recording. Then cells were

perfused with 5 mM ATP in Tyrode solution for 1 min or 5 µM thapsigargin (Tocris,

1138) in Tyrode solution without $CaCl_2$ for 2 min. Once the recording returned to

baseline, 2 µM ionomycin (Sigma-Aldrich) in Tyrode solution was added to reach F_{max}.

A laser scanning confocal microscope (Radiance 2000 MP, Bio-Rad, UK) equipped with

a 40x oil-immersion objective lens (N.A.=1.3) was used to record changes in cytosolic

$[Ca^{2+}]_{cyt}$. Fluo-4 recordings were acquired in line-scan mode (3 ms per scan; pixel size

0.12 µm). All images were analyzed using ImageJ software (NIH, USA). The $[Ca^{2+}]_{cyt}$ was calculated by the following formula: $[Ca^{2+}]_{cyt} = (F_0 - F_{min})/(F_{max} - F_{min})$, where F_0 was the Fluo-4 fluorescence; F_{max} and F_{min} were the fluorescence levels at 3 mM Ca^{2+}/ionomycin and at the lowest baseline recording, respectively. The calcium-induced calcium release was calculated as the summation of the area under the curve for 150 seconds for ATP and 900 seconds for thapsigargin reported in arbitrary units.

To measure $[Ca^{2+}]_{mito}$, either control or VMP1 KO THP-1s were transduced with retroviral vector containing a pMSCVpuro-Mito-Pericam construct as described above [294]. pMSCVpuro-Mito-Pericam was a gift from Björn Stork (Addgene plasmid #87381, http://n2t.net/addgene:87381; RRID:Addgene_87381). THP-1s were differentiated in delta T dishes (Bioptechs, 04200417B). Prior to imaging, the media was replaced by Tyrode solution (NaCl 135 mM; KCl 4 mM; $CaCl_2$ 3 mM; $MgCl_2$ 1 mM; glucose 10 mM; HEPES 10 mM; pH 7.4). Images were acquired using the 60x lens with the EMCCD camera. Cells were excited at 380 nm (DAPI excitation) or 495 nm (FITC excitation) and emission was recorded at 510 nm [295]. Baseline recordings were taken for approximately 30 sec prior to the addition of 5 mM ATP. Five points were taken per dish, and recordings were taken every 8 sec for about 5 min. All images were analyzed using ImageJ software (NIH, USA). The influx rate was quantified for each cell by using the slope of the linear fit of the fluorescence change during 15 sec following the addition of ATP. The efflux rate was quantified for each cell using the slope of the linear fit of a 50 sec period after calcium levels started to decline.

MitoTracker Live Cell Imaging.

To assess mitochondrial mass and mitochondrial membrane potential, THP-1s were differentiated in delta T dishes (Bioptechs, 04200417B) and treated as described above. Following treatment, cells were incubated with a final concentration of 100 nM MitoTracker Red CMXRos (Invitrogen, M7512), 50 nM MitoTracker Green FM (Cell Signaling Technology, 9074S), and 67 ng/mL Hoechst 33342 (ImmunoChemistry Technologies, 639) in serum free RPMI media for 20 min in the incubator. The cells were washed once with FluoroBrite™ DMEM (Gibco, A1896701) supplemented with 10% FBS and 4 mM L-glutamine (Thermo Fisher Scientific, 25030081). Prior to imaging, the heat chamber was set to 37°C and supplemented with 5% CO_2. The exposure conditions were determined based on the treatment conditions where it was expected for there to be the lowest intensity of MitoTracker Red. For each dish, 20 images were acquired with 20 z-stacks per image at 0.5 μm per z-stack for 10 μm total.

Immunofluorescence Imaging.

THP-1s were differentiated onto glass coverslips (Fisherbrand Cover Glasses, 12-545-J, 22 x 60-1) and treated as described above. To visualize mtDNA in the cytoplasm, following treatment, cells were incubated with a final concentration of 100 nM MitoTracker Red CMXRos (Invitrogen, M7512) in serum free RPMI media for 30 min in the incubator. Cells were then washed once with serum free RPMI media then were fixed with 3.7% formaldehyde, methanol free (Polysciences, 04018-1) in 0.1 M piperazine-*N-N*'bis[2-ethanesulfonic acid] (PIPES) buffer for 10 min. Cells were then washed 3x with PBS. The cells were permeabilized with 0.1% saponin in PBS block

solution supplemented with 10% NDS and 0.01% sodium azide and incubated with mouse anti-DNA clone AC-30-10 (1:25, Millipore Sigma; CBL186) for 1 h at RT. Cells were then washed 3x with PBS. Cells were then incubated with 100 ng/mL Hoechst 33342, 488 conjugated donkey anti-mouse (1:300; Jackson ImmunoResearch Laboratories, 715-545-150), and phalloidin-iFluor 647 reagent (1:1000, Abcam; ab176759) for 20 min at RT. Cells were then washed 3x with PBS and were mounted onto coverslips. For each coverslip, 15 images were acquired with 20 z-stacks per image at 0.5 µm per z-stack for 10 µm total.

To visualize LC3/LAMP1, cells were fixed with 3.7% formaldehyde, methanol free in 0.1 M PIPES buffer for 10 min. Cells were then washed 3x with PBS. The cells were permeabilized with 0.1% saponin in PBS block solution supplemented with 10% NDS and 0.01% sodium azide and incubated with rabbit anti-LAMP1 (1:1000, Abcam; ab24170) and mouse anti-LC3B (E5Q2K) (1:300, Cell Signaling; 83506) for 1 h at RT. Cells were then washed 3x with PBS. Cells were then incubated with 100 ng/mL Hoechst 33342, 488 conjugated donkey anti-mouse (1:300), 647 conjugated donkey anti-rabbit (1:300. Jackson ImmunoResearch Laboratories; 711-605-152) for 20 min at RT. Cells were then washed 3x with PBS and were mounted on coverslips. For each coverslip, 15 images were acquired with 20 z-stacks per image at 0.5 µm per z-stack for 10 µm total.

Wide-field Fluorescence Deconvolution Microscopy.

Cells were imaged on a DeltaVision wide-field fluorescence microscope (Applied Precision, Inc.). It has a digital camera (CoolSNAP HQ2; Photometrics) that was used

for fixed cell imaging, and the gain was set to 4.00. For live cell imaging experiments, an

EMCCD camera (Photometrics) was used, and the gain was set to 700. An oil

immersion Olympus Plan Apo 60x objective lens (N.A.=1.42), Olympus UplanSApo

100x objective lens (N.A. = 1.40), or Olympus LUCPlanFLN 20x objective lens (N.A. =

0.45) were coated with 1.518 refraction index low autofluorescence immersion oil,

Olympus Type F (Fischer Scientific, NC0297589). A 250 watt Xenon Arc lamp was used

to direct excitation lighting from the back of the microscope and focused from below

onto the coverslip held on an Olympus IX-71 stage. Dichroic filter set used the Alexa

setting: FITC excitation: 475/28 Emission: 523/36; A594 excitation: 575/25 emission:

632/30; CY5 excitation: 632/20 emission: 67634; DAPI excitation: 390/18 emission:

435/38. Exposure times varied depending on the type of experiment and staining

conditions.

Image Analysis.

The collected z-stack images were used as reconstructed 3-dimensional MIPs for

analysis with Imaris software (version 7.6.4, Bitplane) specifically the 3-dimensional

masking algorithm function. The same masking algorithm was applied to all images and

conditions of a single experiment to allow for consistent group comparisons using the

Batch Coordinator tool (Imaris, Bitplane). For the MitoTracker live cell assay, the spots

algorithm was built around the MitoTracker Green signal with an estimated diameter of

0.750 µm. For the mtDNA immunofluorescence imaging, a surfaces algorithm was built

around the 647 phalloidin channel with the diameter equal to 20 µm and volume above

78.5 µm^3 to create two new channels one for inside of the cell and one for outside of the

cell. A spots algorithm was then built around the 488 DNA signal with an estimated diameter of 0.400 µm and volume between 0.500 µm^3 and 2.000 µm^3. For the LC3/LAMP1 immunofluorescence imaging, a spots algorithm was built around the 488 LAMP1 signal with an estimated diameter of 0.400 µm and area above 0.100 µm^2. Another spots algorithm was built around the 647 LC3 signal with an estimated diameter of 0.500 µm and area above 0.500 µm^2.

Quantification of Cellular and Cell-free mtDNA using qPCR.

Quantitative PCR was performed to measure both cellular and cell-free mtDNA as described in a published protocol [296]. Briefly, cells were treated as described above then the supernatant and cell pellets were collected. The supernatant was spun down at 1500 rpm for 10 min at 4°C to remove cell debris. The cell pellets were resuspended in 200 µL PBS. DNA was isolated following the manufacturer's protocol from the QIAamp DNA Mini Kit (Qiagen, 51304) except the samples were lysed with buffer AL, mixed by pulse-vortexing, and were incubated at 56°C for 10 min. The DNA was then placed in a bath sonicator for 5 min for supernatant or 10 min for cell lysate. After sonication, the concentration of DNA in each sample was determined and was adjusted to the same concentration. The standard curves were generated as described. The quantity of mtDNA in the supernatant was reported as the absolute copy number per µL. The quantity of mtDNA in the cell lysate was reported as the fold difference using the formula $2^{(-\Delta\Delta C_t)}$.

Lysosome Dysfunction Assay.

THP-1s were differentiated as described above in a clear bottom, black 96-well plate (Thermo Fisher Scientific, 07-200-565). Cells were treated as described previously including 0.1 µM bafiloymycin (Cayman Chemicals, 11038) for 4 h. Following treatment, cells were loaded with Magic Red cathepsin B dye (ImmunoChemistry Technologies, 937) for 30 min based on the manufacturers' protocol. Cells were washed 3x with FluoroBrite™ DMEM (Gibco, A1896701) supplemented with 10% FBS and 4 mM L-glutamine (Thermo Fisher Scientific, 25030081). The fluorescence signal was measured using a 528 nm excitation wavelength and 628 nm emission wavelength on a Synergy HTX Multi-Mode plate reader (BioTek Instruments) with Gen5 software. The background signal of a well containing cells without dye was subtracted from each experimental well. Graphed data were normalized to untreated control cells.

Statistical Analysis.

Data were analyzed using GraphPad Prism 5.0. Unless otherwise stated, graphs were presented as the mean ± SEM of at least three independent experiments. Statistical differences were calculated using one-way or two-way ANOVA with Bonferroni post-test, repeated measures ANOVA with Bonferroni post-test, repeated measures ANOVA with Dunnett's multiple comparison test, or two-tailed unpaired t-test as indicated in the figure legends. P-values < 0.05 were considered significant and were designated by: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CHAPTER THREE

RESULTS

SECTION ONE: THE UNCONVENTIONAL SECRETION OF GAL-3 FOLLOWING
INFLAMMATORY STIMULATION

Rationale.

Galectins, a family of proteins that recognize β-galactosides, have diverse

functions that notably contribute to cell activation, proliferation, and apoptosis [297-299].

Interestingly, several galectins modulate processes of disease pathology and

progression such as inflammation [135, 300-302]. Of interest here, gal-3 is widely

expressed in inflammatory cells and contributes to numerous inflammatory conditions

[141, 146, 303-305]. Gal-3 can be released from macrophages and microglial cells,

resident macrophages in the CNS, to propagate inflammatory responses in Alzheimer's

disease and others [135, 146, 306]. Depending on the cellular environment, galectins

can be found both inside and outside of the cell with their localization relating to their

function [131]. These proteins lack a classical leader signal resulting in unconventional

secretion from cells, yet the underlying mechanisms directing gal-3 release from cells

are poorly characterized [131]. Understanding how gal-3 is released and how its

structure is involved will be instrumental in developing targeted therapeutics which can

promote gal-3 function that is neuroprotective of which there are currently no candidates

in trial.

Several studies have aimed to understand the unconventional secretion of other leaderless proteins such as IL-1β and high motility group box 1 (HMGB1). These proteins can be released by secretory autophagy via EVs or they can directly translocate across the PM with some evidence suggesting that gal-3 can also be secreted through these mechanisms. The secretory pathway utilized by gal-3 may depend on the initiating signal [270, 307, 308]. We and others have previously shown that gal-3 localizes to damaged lysosomes and endosomes suggesting release in EVs upon cellular stress [304, 309-312]. Other potential signals include inflammatory responses driven by activation of TLRs and inflammasomes [97]. Conflicting data show that macrophages classically activated by TLR agonists can release either more or less gal-3 [135, 148, 308, 313]. Other inflammatory stimuli may also determine gal-3 release as inflammasome activation has been found to promote fusion of a secretory lysosome with the PM to release the inflammatory mediators, caspase-1 and IL-1β [314, 315]. However, TLR and inflammasome activation have also been found to induce autophagy which in some cases resulted in reduced IL-1β release suggesting at least one mechanism that may influence gal-3 release [316-319]. These conflicting observations may be explained by posttranslational modifications or differences in the localization of gal-3 in the cell upon activation by different inflammatory stimuli, but these hypotheses have yet to be rigorously tested [308].

Some data suggest that gal-3 is predominately secreted outside of EVs by directly crossing the plasma membrane [133, 311]. One possible mechanism for direct

translocation is through GSDMD pores which form following inflammasome activation as part of pyroptosis, a type of inflammatory programmed cell death [118, 119, 134, 320]. Some data suggests that autophagy can protect infected cells from pyroptosis which may negatively regulate gal-3 release, but this idea has not yet been tested [321]. Additionally, the protease, caspase-1, is activated by the inflammasome, and several studies have shown that even proteins such as HMGB1 and IL-1α that are not substrates of caspase-1 are dependent on the active enzyme for their release, yet why there is this dependence is not understood [307, 322-324]. Proteomics studies have found that gal-3 can bind to caspase-1, but whether caspase-1 influences gal-3 release has not yet been determined [307]. Thus, the underlying mechanisms that delineate how gal-3 is secreted following activation by various inflammatory stimuli need to be well-characterized. The central hypothesis of this section is that inflammatory signals selectively induce gal-3 release through either secretory autophagy or direct translocation across the PM through GSDMD pores in human macrophage-like cells.

Results.

Gal-3 is Predominantly Released through GSDMD Pores under Inflammatory Conditions.

To determine whether GSDMD is required for the release of gal-3 under inflammatory conditions, we utilized CRISPR-Cas9 to knockout GSDMD in THP-1 cells, a human macrophage-like cell line. The knockout was verified by western blot (Fig. 11A). To induce inflammatory responses in these cells, differentiated THP-1s were treated with LPS which activates TLR4 and upregulates proinflammatory genes then the

cells were treated with ATP which activates the inflammasome resulting in formation of GSDMD pores.
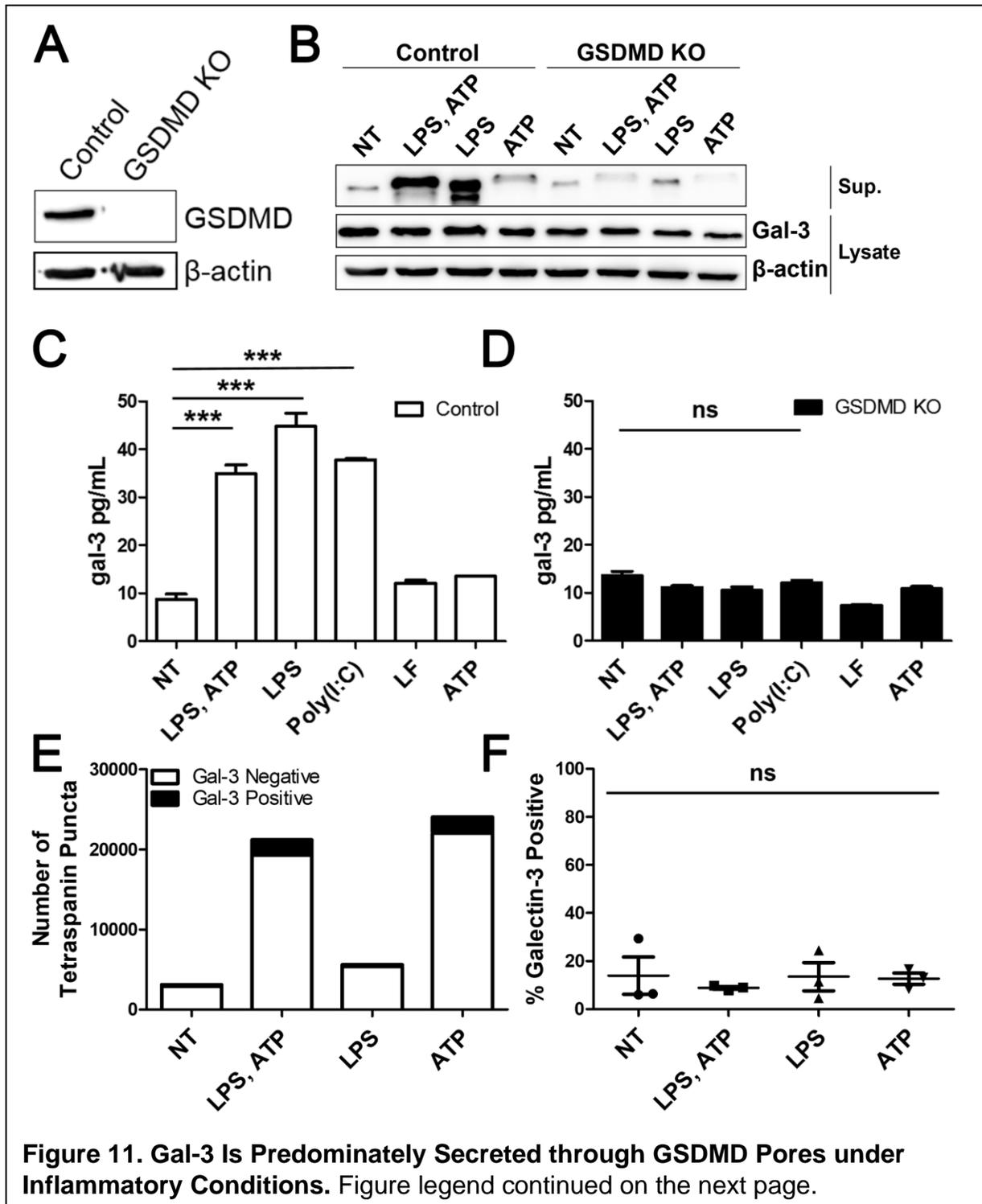
Figure 11. Gal-3 Is Predominately Secreted through GSDMD Pores under Inflammatory Conditions. Figure legend continued on the next page.

Figure 11. Gal-3 Is Predominately Secreted through GSDMD Pores under Inflammatory Conditions. THP-1 cells were depleted of GSDMD by using CRISPR-Cas9 genome editing. A) Protein expression in the depleted cells was confirmed by western blotting. B) Differentiated control or GSDMD KO THP-1s were treated with 100 ng/mL LPS for 4 h ± 5 mM ATP for 30 min or 5 mM ATP alone for 30 min. Supernatant and lysates were collected and probed for gal-3 protein levels. C-D) Differentiated control or GSDMD KO THP-1s were treated with 100 ng/mL LPS for 4 h ± 5 mM ATP for 30 min, 50 µg/mL poly(I:C) for 4 h, Lipofectamine2000 as a transfection control, or 5 mM ATP for 30 min. Supernatant was collected at the same time for all treatments, and gal-3 released into the supernatant was measured by ELISA. Data are displayed for C) control cells and D) GSDMD KO cells. E) Immunofluorescence microscopy was performed to visualize EVs released into the supernatant from untreated or LPS and ATP treated control or GSDMD KO cells. The EVs were stained with a tetraspanin cocktail and gal-3. The number of tetraspanin puncta that were either positive or negative for gal-3 are displayed. F) Quantification of the percentage of tetraspanin puncta that were positive for gal-3. Data are shown as mean ± SEM and are either representative of or the average of at least three independent experiments. Statistical differences were calculated with two-way ANOVA followed by Bonferroni post-test. For all statistical tests, *, **, ***, p < 0.05, 0.01, and 0.001, respectively.

LPS alone can also noncanonically activate the inflammasome to form GSDMD pores

[8]. To measure gal-3 release in the supernatant from GSDMD KO THP-1s, cells were

treated with inflammatory stimuli then the supernatant was collected. Treatment of THP-

1s with LPS and ATP or LPS alone resulted in detection of gal-3 in the supernatant by

western blot, but in treated GSDMD KO THP-1s, there was a decrease in the release of

gal-3 from these cells (Fig. 11B). The amount of gal-3 in the

lysates was comparable between the control and GSDMD KO cells and did not change

with the addition of inflammatory stimuli. In addition, the release of gal-3 from control or

GSDMD KO THP-1s was quantified by ELISA. In this experiment, cells also were

treated with poly(I:C) which can induce NLRP3 inflammasome activation and formation

of GSDMD pores [325]. There was an increase in the release of gal-3 from control THP-

1s treated with LPS and ATP, LPS alone, and poly(I:C) (Fig. 11C). The release of gal-3 from treated GSDMD KO THP-1s was reduced to levels comparable to the no treatment control suggesting that the release of gal-3 under these inflammatory conditions was dependent on GSDMD pores (Fig. 11D).

Given our previous reports, we hypothesized that gal-3 also can be released in EVs under inflammatory conditions [291]. To determine whether gal-3 is released in EVs, we utilized an immunofluorescence-based assay previously developed in our lab named EV multiplex analysis of colocalization (EV-MAC) [326]. Here EVs were stained with anti-CD9, anti-CD63, and anti-CD81 which all recognize tetraspanins which are transmembrane proteins that are considered a marker of EVs [327]. It is thought that CD9, CD63, and CD81 are unevenly distributed amongst EVs which is why here a tetraspanin cocktail of all three was utilized. A spots algorithm was built around the tetraspanins which allowed us to quantify the number of EVs released under different conditions and ask what percentage of tetraspanin puncta were also positive for gal-3. There was a large increase in the number of tetraspanin puncta that were released from LPS and ATP or ATP only treated THP-1s (Fig. 11E). These results were consistent with a previous study that found that stimulation of the P2X7R by ATP resulted in an increase in the shedding of EVs [328]. To determine whether gal-3 is released in EVs under inflammatory conditions, we quantified the percentage of tetraspanin puncta that were positive for gal-3. The data showed that there was no difference in the relative percentage of EVs containing gal-3 under inflammatory conditions (Fig. 11F). On average, about 10-15% of EVs contain gal-3. Even though there was a large increase in

the number of EVs released under certain inflammatory conditions, the relative amount containing gal-3 was the same under different conditions. Collectively, these data suggested that gal-3 can be released in EVs although it was predominantly released through GSDMD pores under these inflammatory conditions.

Discussion.

In this chapter, we elucidate the mechanism for how gal-3 is released following activation of the cells with inflammatory stimuli. Initially, we hypothesized that gal-3 was likely released in EVs because there was evidence that this was a predominant mechanism of release in our work and others [279, 291]. Rupture of vesicular membranes exposes intralumenal glycans which are recognized by galectins. Autophagic proteins are recruited and galectins are introduced to the autophagic-lysosomal pathway which ultimately results in unconventional secretion [291]. Alternatively, recent work demonstrated that gal-3 can be released through GSDMD pores formed as a result of inflammasome activation in BMDMs [134]. Numerous examples in the literature indicate that there are differences in the inflammatory responses of human and murine derived macrophages [329-331]. Our data in differentiated THP-1s, a human model macrophage-like cell line, demonstrated that gal-3 can be released both in EVs as well as through GSDMD pores. Even though the number of EVs released under inflammatory conditions increased greatly with ATP activation the relative percentage of EVs containing gal-3 was comparable between cells treated with inflammatory stimuli compared to control. The predominant mechanism for gal-3 release following activation by LPS and ATP, LPS alone, and

poly(I:C) alone was through GSDMD pores. It may be interesting in the future to leverage the EV-MAC assay to analyze the contents of the EVs released from macrophages treated with inflammatory stimuli since the number of EVs increased greatly with ATP activation [326]. The advantage of the EV-MAC approach is that it allows for the contents of individual EVs to be determined in contrast to the previous approaches that use western blotting to identify the bulk contents of EVs. Overall, these data suggested that gal-3 was predominantly released through GSDMD pores in response to canonical inflammatory stimuli.

In the next section, we will assess the potential regulatory role of VMP1 in inflammatory responses. Now that we have demonstrated that gal-3 was released through GSDMD pores downstream of inflammasome activation, we can compare patterns of gal-3 and IL-1β secretion from VMP1 KO cells. It can help us to determine whether the response in VMP1 KO cells is due to changes in inflammasome activation or potentially other mechanisms.

SECTION TWO: VMP1 RESTRICTS INFLAMMASOME ACTIVATION THROUGH ITS MODULATION OF SERCA ACTIVITY AND AUTOPHAGY

Rationale.

VMP1 was first characterized in acute pancreatitis models, an inflammatory disease that results in significant cell death [10]. More recently, it has been identified that VMP1 expression is associated with the severity of cancer and inflammatory bowel disease [332, 333]. With connections to inflammatory disease, we aimed to understand how VMP1 may regulate inflammatory signaling. In the canonical inflammatory pathway,

two signals are required: the first signal induces upregulation of proinflammatory gene expression, and the second signal activates a multiprotein complex specifically the NLRP3 inflammasome. Activation of these pathways ultimately results in the release of inflammatory mediators such as IL-6, IL-1β, and gal-3. Interestingly, both pathways are activated at least in part by changes in intracellular calcium levels which are known to be influenced by VMP1 [188, 334, 335], yet a regulatory role for VMP1 in these responses has not yet been identified. Intriguingly, while there are many known activators of inflammasomes, questions persist about which specific signals are required for inflammasome activation particularly when more than one signal is present in the same cell. Thus, identifying and characterizing a novel regulator of these pathways will be instrumental in being able to specifically target and successfully modulate inflammatory responses in disease.

Surprisingly, better characterized VMP1 orthologs have pleiotropic phenotypes suggesting that VMP1 likely regulates several cellular processes including protein secretion [16-18]. Of interest here was characterizing how VMP1 may affect the release of proinflammatory cytokines and gal-3 following inflammatory activation of macrophages. Existing data suggested that both damaged mitochondria and calcium fluxes can activate inflammatory pathways, but how VMP1 modulation of these signals may impact cellular inflammatory responses was not yet understood. Perhaps given its known functions, VMP1 may regulate these responses by degrading damaged mitochondria and buffering cytoplasmic Ca^{2+} concentrations [5, 150]. Thus, the underlying mechanisms that delineate how VMP1 regulates inflammatory signaling

pathways need to be well-characterized. The central hypothesis of this section is that VMP1 modulates cytoplasmic Ca^{2+} concentrations and mediates the degradation of damaged mitochondria to negatively regulate inflammasome activation and the release of proinflammatory cytokines and gal-3.

Results.

VMP1 Negatively Regulates the Release of Proinflammatory Molecules.

To determine whether VMP1 affects the secretion of proinflammatory molecules, we utilized CRISPR-Cas9 to knockout VMP1 in THP-1s, a human model macrophage like cell line [12, 336]. The knockout was verified by western blot (Fig. 12A). To induce canonical inflammatory signaling, differentiated THP-1s were treated with LPS to activate TLR4 and induce the upregulation of inflammatory cytokine gene expression and then ATP to activate the inflammasome. To induce noncanonical activation of the inflammasome, LPS alone was used [337]. Transfected poly(I:C), representative of cytosolic dsRNA produced during viral infection, was also used to activate the NLRP3 inflammasome [325]. VMP1 expression does not change even when cells are treated with inflammatory stimuli (Fig. 13A). To initially assess whether there are differences in inflammatory responses in VMP1 KO cells, IL-1β release was measured. Treatment of VMP1 KO THP-1s with LPS and ATP resulted in increased secretion of IL-1β compared to treated control cells suggesting that there may be increased inflammasome activation in VMP1 KO cells (Fig. 12B). Since it was recently observed that PD patients have reduced expression of VMP1 in PBMCs, we wanted to ask whether primed VMP1 KO macrophages treated with α-syn release more IL-1β.

Figure 12. VMP1 Negatively Regulates the Release of Proinflammatory Cytokines and Gal-3 under Inflammatory Conditions. THP-1 cells were depleted of VMP1 by using CRISPR-Cas9 genome editing. A) Protein expression in the depleted cells was confirmed by western blotting. B) Differentiated control or VMP1 KO THP-1s were treated with 100 ng/mL LPS for 4 h ± 5 mM ATP for 30 min, 50 µg/mL poly(I:C) for 4 h, Lipofectamine2000 as a transfection control, or 5 mM ATP for 30 min. IL-1β released into the supernatant was measured by ELISA. C) Differentiated control or VMP1 KO THP-1s were treated with 100 ng/mL LPS for 4 h then 1 µM α-syn fibrils for 24 h, 100 ng/mL LPS for 4 h then 5 mM ATP for 30 min, 1 µM α-syn fibrils for 24 h, or 100 ng/mL LPS for 4 h. Supernatant was collected at the same time for all treatments, and IL-1β released into the supernatant was measured by ELISA. D) Similar experiments to B were performed to measure gal-3 release. E) Similar experiments to B were performed to measure IL-6 release. Data are shown as mean ± SEM and are either representative of or the average of at least three independent experiments. Statistical differences were calculated with two-way ANOVA followed by Bonferroni post-test. For all statistical tests, *, **, ***, $p < 0.05$, 0.01, and 0.001, respectively.

Under these conditions, supernatant was collected after 24 hrs. There was elevated

basal IL-1β release from VMP1 KO cells compared to control cells suggesting that

VMP1 KO cells were already more proinflammatory without the addition of inflammatory stimuli (Fig. 12C). Primed VMP1 KO cells treated with α-syn released more IL-1β than control cells (Fig. 12C). These data suggested that PD patients with decreased VMP1 expression may have an increased basal level of inflammation as well as exacerbated inflammatory responses to protein aggregates characteristic of the disease.

Previous reports and our data above suggested that gal-3 was released through GSDMD pores under inflammatory conditions. As expected VMP1 KO cells treated with LPS and ATP, LPS alone, and poly(I:C) alone had increased release of gal-3 which was consistent with the results for IL-1β release (Fig. 12D). Additionally, VMP1 KO cells treated with ATP alone had increased gal-3 release compared to treated control cells (Fig. 12D). Given that the autophagosome-lysosome pathway (ALP) was impaired in VMP1 KO cells and that there was an increase in EV release with ATP treatment (Fig. 11E), perhaps that is why there was increased gal-3 release from ATP treated VMP1 KO cells although this would need to be tested directly [5, 31]. Interestingly, VMP1 KO cells had increased IL-6 release from cells treated with LPS and ATP, LPS alone, and poly(I:C) alone (Fig. 12E). IL-6 gene expression is upregulated following TLR activation, yet its release occurs independently of inflammasome activation. VMP1 negatively regulated the expression of proinflammatory cytokines, proIL-1β and IL-6, but had no effect on the expression of gal-3 (Fig. 13B, 13C, and 13D). Perhaps increased expression of IL-6 in VMP1 KO cells was sufficient to result in its increased release, but that would need to be tested. Overall, increased release of IL-6 when cells were treated

with LPS and ATP, LPS alone and poly(I:C) alone suggested that VMP1 may promote

hypersensitive TLR signaling (Fig. 12E).

Figure 13. VMP1 Negatively Regulates the Expression Levels of Canonical Proinflammatory Cytokines but has no Effect on Gal-3 Expression in Macrophages. Differentiated THP-1s were treated with various inflammatory stimuli and lysates were collected for qPCR. A) VMP1 gene expression in treated WT THP-1s. Cell lysates were collected following treatment with inflammatory stimuli and gene expression changes for B) proIL-1β, C) IL-6 and D) lgals3 were measured using qRT-PCR with gene specific primers. Fold change in mRNA gene expression compared with the NT control of either control or VMP1 KO THP-1s are presented. Data in A) are represented as the mean ± SEM of three independent experiments. The rest of the data are presented as the mean +/- error for samples run in triplicate representative of at least three independent experiments.

Second, increased IL-1β release from LPS and ATP treated VMP1 KO macrophages suggested that dysregulation in VMP1 KO cells may induce increased inflammasome activation (Fig. 12B). Therefore, these data suggested that VMP1 is a novel regulator of inflammatory signaling and that it may have this effect either by 1) creating a state that allows for hyperactivation of TLR signaling and/or 2) promoting dysregulation such that conditions promote increased inflammasome activation. Collectively, these data suggested that VMP1 negatively yet differentially regulated the release of IL-1β, IL-6, and gal-3 depending on the inflammatory stimuli and that VMP1 KO cells have elevated basal inflammatory responses.

VMP1 KO Cells have Increased Caspase-1 and Caspase-3 Activation Depending on the Stimuli.

To determine whether VMP1 KO cells have increased inflammasome activation, we employed a fluorochrome-labeled inhibitors of caspases assay (FLICA) to measure active caspase-1. Treatment of VMP1 KO cells with LPS and ATP and ATP alone resulted in an increase in FLICA signal compared to control cells suggesting that when cells were treated with these inflammatory stimuli there was an increase in caspase-1 activation (Fig. 14A). To assess changes in inflammasome activation over time, we measured biosensor activation where upon inflammasome activation, a caspase-1 consensus sequence is cleaved resulting in active firefly luciferase [290]. We observed that in VMP1 KO cells treated with LPS and ATP there was increased biosensor activation at 4 h which is right before the addition of ATP and at 7.5 h which is several hours after the addition of ATP (Fig. 14B). Similar trends were observed for cells treated with LPS alone (Fig. 14C). There was no difference in biosensor activation between

VMP1 KO and control cells treated with poly(I:C) which was consistent with the FLICA data (Fig. 14D). Since there was no difference in inflammasome activation in poly(I:C) treated cells, there might be an inflammasome independent reason for why there was increased release of gal-3 in VMP1 KO cells compared to control cells. Interestingly, there was increased inflammasome activation in VMP1 KO cells treated with ATP alone even at time points prior to the addition of the DAMP supporting the idea that there were elevated basal inflammatory responses in VMP1 KO cells compared to control cells (Fig. 14E). Collectively, these data suggested that there was increased caspase-1 activation in VMP1 KO cells treated with LPS and ATP.

To determine whether this increase in inflammasome activation was specific to the NLRP3 inflammasome, we pretreated cells with MCC950, a specific inhibitor of the NLRP3 inflammasome, prior to the addition of ATP then measured caspase-1 biosensor activation [338]. When either LPS and ATP treated VMP1 KO or control cells were pretreated with MCC950, there was a decrease in caspase-1 activation comparable to the no treatment control (Fig. 14F). These data suggested that it was specifically the NLRP3 inflammasome that was being activated in VMP1 KO cells. To assess whether there was increased cell death in treated VMP1 KO cells compared to control, we probed for cleaved caspase-3, a marker of apoptotic cell death, by western blot. An increase in cleaved caspase-3 was detected in VMP1 KO cells treated with LPS and ATP and poly(I:C) as well as a slight increase with LPS treatment alone indicating that there was induction of cell death (Fig. 14G). Additionally, LDH release was quantified as an indicator of pyroptosis [339].
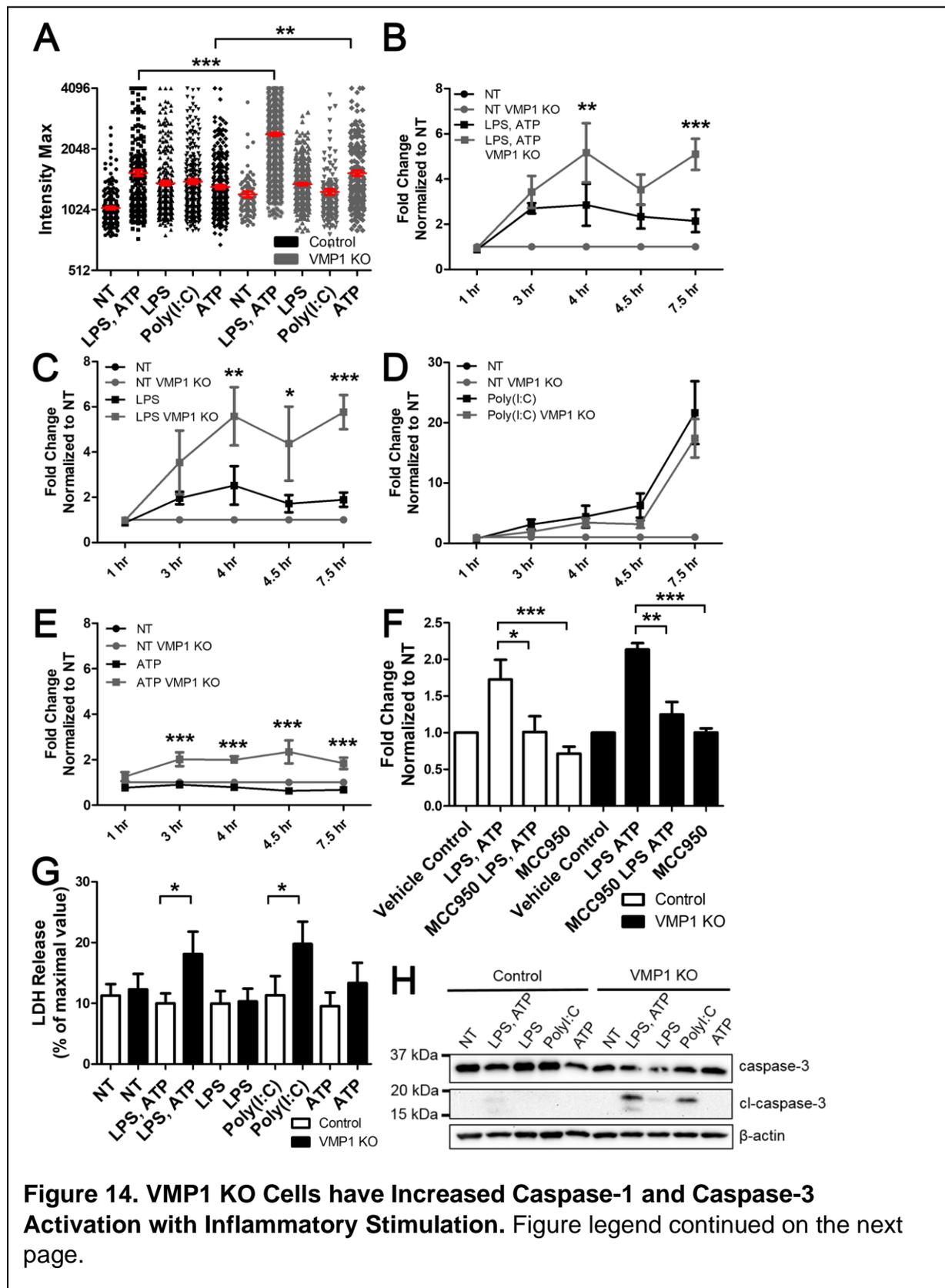
Figure 14. VMP1 KO Cells have Increased Caspase-1 and Caspase-3 Activation with Inflammatory Stimulation. Figure legend continued on the next page.

Figure 14. VMP1 KO Cells have Increased Caspase-1 and Caspase-3 Activation with Inflammatory Stimulation. Differentiated control or VMP1 KO cells were treated with LPS and ATP, LPS, poly(I:C), and ATP. A) Caspase-1 activation was assessed by confocal microscopy using a FLICA assay. Data are represented as the intensity max per cell from 10 images per treatment. B-E) Control or VMP1 KO THP-1s transduced with the C7 biosensor were activated with B) LPS and ATP, C) LPS, D) poly(I:C), and E) ATP and luminescence was measured at 1 h, 3 h, 4 h, 4.5 h, and 7.5 h following treatment. F) THP-1s were treated with an NLRP3 inflammasome inhibitor, MCC950, then caspase-1 activation was measured with the C7 biosensor. G) Caspase-3 and cleaved caspase-3 were measured by western blotting. H) THP-1s were treated with inflammatory stimuli then cell death was measured by LDH assay. Data are shown as mean ± SEM and are either representative of or the average of at least three independent experiments. Statistical differences were calculated with two-way ANOVA followed by Bonferroni post-test or repeated measures ANOVA followed by Bonferroni post-test. For all statistical tests, *, **, ***, $p < 0.05$, 0.01, and 0.001, respectively.

An increase in LDH release was measured from VMP1 KO cells treated with LPS and

ATP and poly(I:C) which corresponded with the amount of cleaved caspase-3 (Fig.

14H). Collectively, these data suggested that in VMP1 KO cells there was increased

caspase-1 activation specifically through the NLRP3 inflammasome along with

increased cell death corresponding to treatment with specific inflammatory stimuli.

VMP1 KO Cells have Depleted ER Ca^{2+} Stores but Elevated Intracellular [Ca^{2+}] with ATP Activation.

Previous work in the literature suggests VMP1 can regulate the activity of the ER

calcium channel SERCA by stabilizing it in its active form and directly preventing

interaction with its inhibitory binding partners [5]. This study primarily focused on

characterizing how this function of VMP1 influenced membrane contacts at the ER, but

it did not address how this interaction influenced other calcium sensitive pathways in the

cell [5]. In particular, when cells are activated with ATP, there is K$^+$ efflux through the

P2X7 receptor that is accompanied by an influx of Ca^{2+} which may be perturbed in

VMP1 KO cells that have diminished SERCA activity [340]. To measure changes in intracellular $[Ca^{2+}]$, a high-affinity Ca^{2+} indicator, Fluo-4 AM, was used with live cell imaging. To assess whether there were changes in $[Ca^{2+}]_{cyt}$ when cells were activated with ATP, differentiated VMP1 KO or control cells were perfused with a solution of ATP in Tyrode solution containing 3 mM Ca^{2+}. There was a longer response to ATP treatment in VMP1 KO cells compared to control (Fig. 15A). The average calcium induced calcium release (CICR) area was calculated for several cells. The CICR area describes a general mechanism whereby Ca^{2+} triggers the release of further Ca^{2+} from intracellular stores. The CICR area for ATP-treated VMP1 KO was greater than control cells (Fig. 15B). Previous studies have demonstrated that VMP1 can regulate the activity of SERCA, a Ca^{2+}-ATPase [5]. To measure the relative amount of Ca^{2+} stored in the ER of VMP1 KO versus control cells, differentiated THP-1s were perfused with a solution containing thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase, in Tyrode solution without Ca^{2+}. These data showed that there was a small amount of Ca^{2+} in the VMP1 KO cells that was rapidly released upon the addition of thapsigargin compared to control cells that comparatively have a sustained release of Ca^{2+} (Fig. 15C-D). To test whether the VMP1 regulation of SERCA contributed to inflammasome activation, cells were treated with inflammatory stimuli in the presence of thapsigargin or thapsigargin alone. The data showed that there was increased caspase-1 activation in control THP-1s when cells were treated with LPS and ATP or ATP alone in the presence of thapsigargin suggesting that inhibition of SERCA was contributing to the exacerbated inflammatory phenotype observed in VMP1 KO

cells (Fig. 15E).

Figure 15. VMP1 KO Cells have Depleted ER Ca²⁺ Stores, but Increased Intracellular [Ca²⁺] with ATP Treatment. Figure legend continued on the next page.

Figure 15. VMP1 KO Cells have Depleted ER Ca^{2+} Stores, but Increased Intracellular $[Ca^{2+}]$ with ATP Treatment. Either control or VMP1 KO THP-1s were perfused with Tyrode solution containing 3 mM Ca^{2+} for 1 min then were either perfused with A-B) ATP or C-D) thapsigargin prior to adding F_{max} consisting of Tyrode solution with 3 mM Ca^{2+} and ionomycin. A,C) Representative traces of the normalized fluorescence intensity of individual cells. B,D) Average calcium-induced calcium release (CICR) area under the curve of individual cells for either B) 150 sec or D) 900 sec. E) Caspase-1 activation assessed using confocal microscopy and a FLICA assay. Data are represented as the intensity max of individual cells for a given treatment. Statistical differences were calculated with two-tailed unpaired t-tests or one-way ANOVA followed by Bonferroni post-test. For all statistical tests, *, **, ***, $p <$ 0.05, 0.01, and 0.001, respectively.

When VMP1 KO cells were treated with LPS and ATP in the presence of thapsigargin, there was a decrease in caspase-1 activation which might be explained by the depleted ER store of Ca^{2+} prior to the addition of ATP. Previous studies have shown that Ca^{2+} mobilization can contribute to inflammasome activation [189]. To determine whether Ca^{2+} fluxes alone influence inflammasome activation, cells were treated with thapsigargin. The results showed a slight increase in FLICA signal in either control or VMP1 KO cells treated with thapsigargin suggesting that Ca^{2+} release from the ER has a modest effect on inflammasome activation (Fig. 15E).

To further determine whether Ca^{2+} mobilization affects inflammasome activation and cytokine release, differentiated THP-1s were incubated with chemical compounds that deplete extracellular Ca^{2+} or inhibit release of Ca^{2+} from inside of the cell. There was an increase in the release of IL-1β and gal-3 when VMP1 KO cells were incubated with EGTA and thapsigargin or EGTA and ionomycin (Fig. 16A-B). This increase was likely due to the dysregulated response to changes in Ca^{2+} in VMP1 KO cells that then induced K^+ efflux which is a known activator of the inflammasome [341].

Figure 16. VMP1 KO Cells Release More Proinflammatory Molecules when Extracellular [Ca^{2+}] are Depleted Prior to the Release of Intracellular Ca^{2+} Stores. THP-1s were incubated with chemical compounds that deplete either extracellular or intracellular Ca^{2+} stores. ELISAs were used to measure the amount of either A) IL-1β or B) gal-3 released into the supernatant. Cells were lysed to measure caspase-1 activation using the biosensor in C). Data in A) and B) are representative of at least three independent experiments. Data are presented as the mean ± SEM. Data in C) are presented as the mean ± SEM of at least three independent experiments. Statistical differences were calculated using either one-way or two-way ANOVA with Bonferroni post-test. For all statistical tests, *, **, ***, p < 0.05, 0.01, and 0.001, respectively.

Caspase-1 activation measured by the biosensor corresponded with these data where there was a modest increase in caspase-1 activation with thapsigargin treatment, but there was a larger increase in this activation when extracellular Ca^{2+} was depleted with EGTA (Fig. 16C). Taken together, these data suggested that when VMP1 was not present to regulate SERCA, there were elevated levels of intracellular Ca^{2+} following inflammatory stimulation. In WT cells, treatment with thapsigargin increased caspase-1 activation suggesting that at least in part there was increased inflammasome activation due to inhibition of the SERCA pump. Yet there were likely other changes associated with VMP1 function that also increased inflammasome activation.

VMP1 KO Cells have an Increased Rate of Ca^{2+} Influx into Mitochondria upon ATP Stimulation.

To quantify the effect of VMP1 on mitochondrial buffering of Ca^{2+}, a stable THP-1 cell line expressing the Ca^{2+}-sensitive ratiometric fluorescent protein mito-pericam were generated [294]. This construct consists of a fluorescent $[Ca^{2+}]$ probe that contains a modified permutated EYFP where the C- and N- terminal domains were exchanged and connected by a Gly-rich linker as well as the introduction of the following mutations: F46L, Q69K, H148D, V163A, S175G, and Y203F. This sequence is flanked by calmodulin (CaM) at the C-terminus and by an M13 peptide (the CaM binding site (K566-L591) of human MYLK2) at the N-terminus. This construct has a localization signal for mitochondria and can be excited at different wavelengths depending on how much calcium is present (Fig. 17A and 17B). An increase in $[Ca^{2+}]$ leads to binding of CaM to M13 peptide which results in a conformational change. This structural change results in a shift of excitation from ~410 nm to ~495 nm with the emission peak

remaining the same at ~515 nm. The fluorescence intensity ratio of Ex495/Ex410

increases up to tenfold with elevated [Ca^{2+}]. Live cell imaging was performed with

recordings of the basal level of fluorescence in the DAPI and FITC channels then for a

period after the addition of inflammatory stimuli.
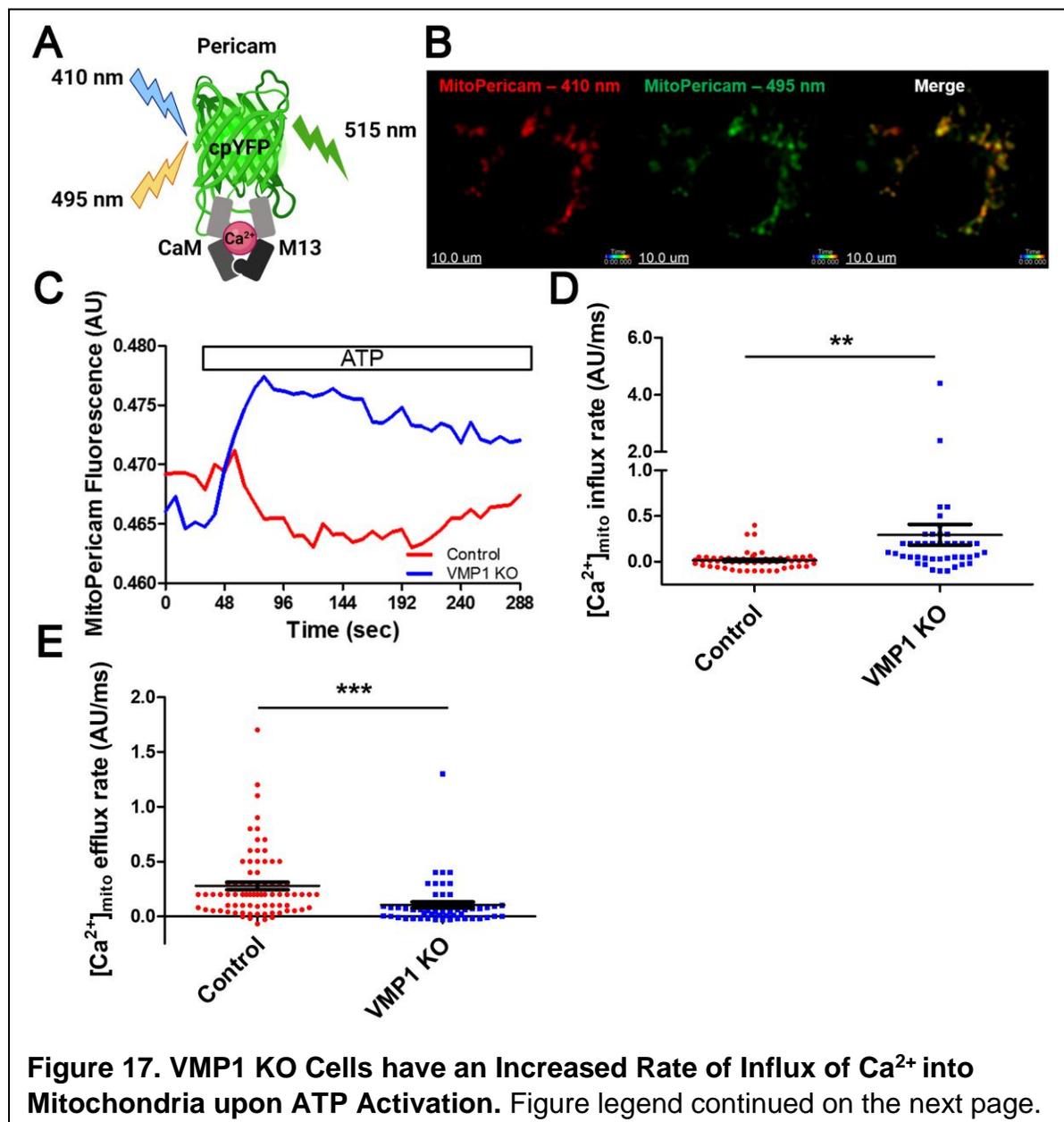
Figure 17. VMP1 KO Cells have an Increased Rate of Influx of Ca^{2+} into Mitochondria upon ATP Activation. Figure legend continued on the next page.

Figure 17. VMP1 KO cells have an Increased Rate of Influx of Ca^{2+} into Mitochondria upon ATP Activation. Differentiated control or VMP1 KO THP-1s were transduced with a pMSCVpuro-Mito-Pericam construct. Cells were excited at 410 nm and 495 nm first to collect baseline recordings then cells were activated with ATP. A) Schematic illustrating how the pMSCVpuro-Mito-Pericam construct works. The ratiometric pericam is a fluorescent $[Ca^{2+}]$ probe that contains a modified permutated EYFP flacked by calmodulin and an M13 peptide which contains the CaM binding site. At low $[Ca^{2+}]$, the construct is excited at 410 nm, but an increase in $[Ca^{2+}]$ facilitates the binding of CaM to the M13 peptide resulting in structural changes that cause a shift of the excitation maximum from ~410 nm to ~495 nm. B) Representative images of cells expressing the mito-pericam construct. C) Representative traces of the ratio of fluorescence intensity at 495 nm/410 nm of individual cells. D) Average $[Ca^{2+}]_{mito}$ influx rate of individual cells over 15 sec. E) Average $[Ca^{2+}]_{mito}$ efflux rate of individual cells over 50 sec. Data are either representative of three independent experiments (C) or the average of individual cells from at least three independent experiments. Statistical differences were calculated with two-tailed unpaired t-tests. For all statistical tests, *, **, ***, $p < 0.05$, 0.01, and 0.001, respectively.

To assess changes in the $[Ca^{2+}]_{mito}$ of VMP1 KO versus control cells upon ATP stimulation, images were acquired by exciting the cells at 410 nm and 495 nm for baseline recordings then ATP was added. The mito-pericam fluorescence intensity was displayed as the ratio of 495 nm/410 nm. Representative traces of the fluorescence intensity of VMP1 KO or control cells demonstrated that upon ATP stimulation, there was an increase in the ratio of the fluorescence intensity for VMP1 KO cells compared to control cells (Fig. 17C). To determine the influx rate of Ca^{2+} into mitochondria, the slope of the line for the fluorescence intensity ratio was determined over a 15 sec interval following the addition of ATP. On average, the $[Ca^{2+}]_{mito}$ influx rate was faster in VMP1 KO cells compared to control cells (Fig. 17D). To determine the efflux rate of Ca^{2+} from mitochondria, the slope of the line for the fluorescence intensity ratio was determined over a 50 sec period after calcium levels started to decline. The efflux rate

of $[Ca^{2+}]_{mito}$ was faster for control cells treated with ATP suggesting that calcium was extruded faster from control cells than VMP1 KO cells (Fig. 17E). Collectively, these data suggested that mitochondrial calcium dynamics were altered in VMP1 KO cells such that there was increased uptake of Ca^{2+} following ATP stimulation as evidenced by an increased influx rate over the same period as control as well as a decreased extrusion rate of Ca^{2+} from the mitochondria following ATP stimulation.

VMP1 KO Cells have More and Smaller Mitochondria that Lose their Membrane Potential with LPS and ATP Treatment.

To characterize mitochondrial fitness, we developed an approach where live differentiated THP-1s were incubated with fluorescent mitochondrial dyes then confocal microscopy images were acquired. Differentiated THP-1s were incubated with MitoTracker Green (MTG) dye and MitoTracker Red CMXRos (CMXRos) dye. MTG is a fluorescent probe that assesses mitochondrial mass. It accumulates in mitochondria independently of the mitochondrial transmembrane potential meaning that it labels mitochondria in living cells. CMXRos is a fluorescent probe that stains mitochondria, but its accumulation is dependent upon the mitochondrial membrane potential. At steady state, macrophages have high mitochondrial polarization, but upon macrophage activation, it is typical to observe a loss in mitochondrial membrane potential. An algorithm was built around the MTG channel using Imaris to allow us to not only measure the membrane potential of individual mitochondria but also to quantify the number of mitochondria per cell and the average mitochondrial volume (Fig. 18A). To further assess the relative amounts of functional or dysfunctional mitochondria, the intensity max of the CMXRos was plotted on the y-axis and the intensity max of the

MTG was plotted on the x-axis. Gridlines were drawn where mitochondria that were

CMXRos and MTG high were considered functional and mitochondria that were

CMXRos low and MTG high were considered dysfunctional.
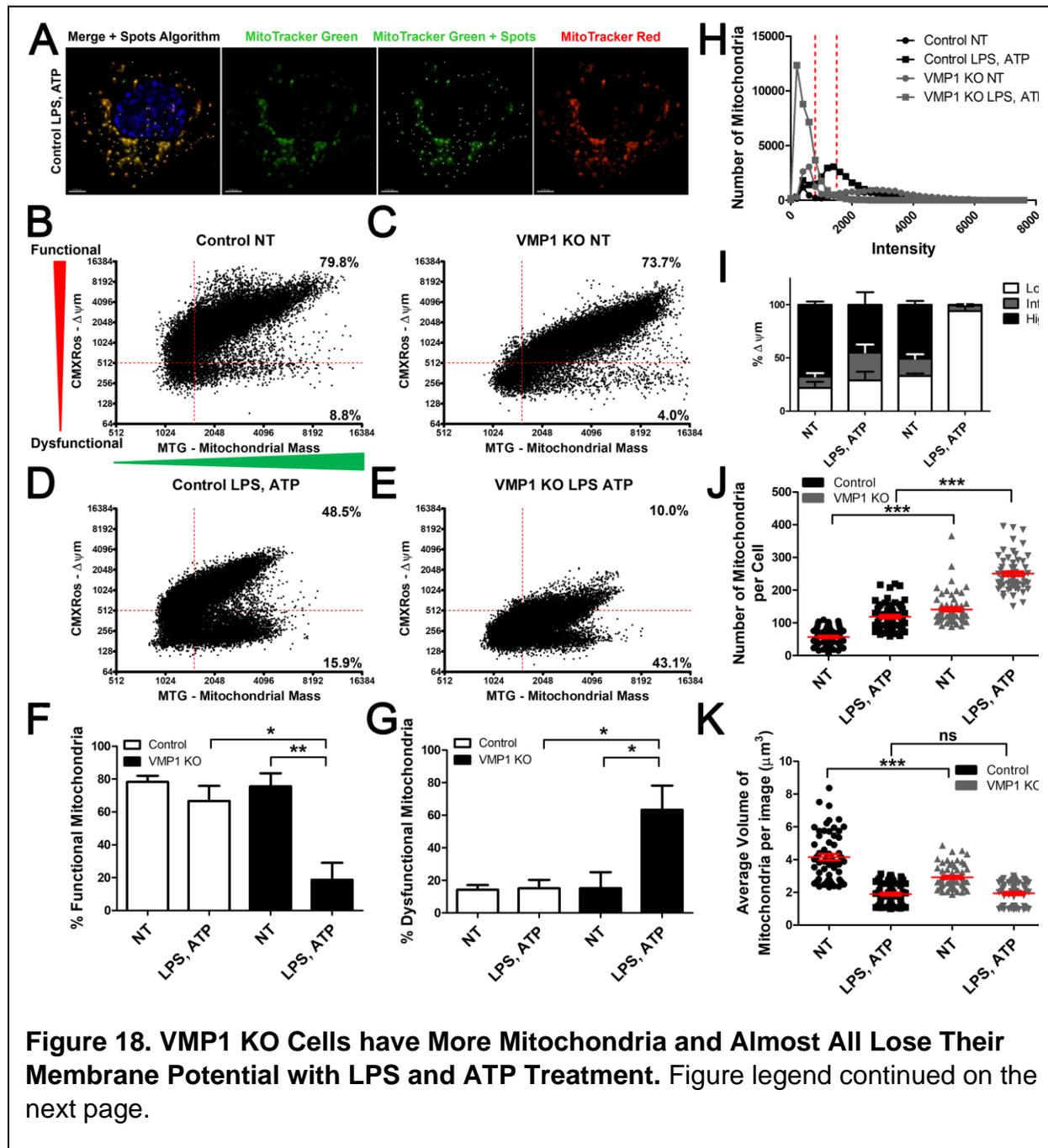
Figure 18. VMP1 KO Cells have More Mitochondria and Almost All Lose Their Membrane Potential with LPS and ATP Treatment. Figure legend continued on the next page.

Figure 18. VMP1 KO Cells have More Mitochondria and Almost All Lose Their Membrane Potential with LPS and ATP Treatment. Control or VMP1 KO cells were left untreated or treated with LPS and ATP. Live cells were stained with CMXRos with its accumulation in the cell dependent on mitochondrial membrane potential and with MTG to assess mitochondrial mass. Imaris was then used to build a spots algorithm around the MTG channel. A) Representative image of treated control THP-1s that were incubated with CMXRos and MTG. B) Quantification of the average number of mitochondria per cell per image. C) Quantification of the average volume of individual mitochondria per image. D-G) Plots of the intensity max of CMXRos vs. MTG for individual mitochondria. Percentages in the upper right quadrant correspond to functional mitochondria and percentages in the lower right quadrant correspond to dysfunctional mitochondria. H) Percentages for CMXRos High/MTG High populations corresponding to functional mitochondria represented as the mean ± SEM for three independent experiments. I) Percentages for CMXRos Low/MTG High populations corresponding to dysfunctional mitochondria represented as the mean ± SEM for three independent experiments. J) Representative histogram depicting the number of mitochondria in each bin of intensity max for the CMXRos channel. K) Plot showing the percentage of mitochondria that have low, intermediate, and high mitochondrial membrane potential. Statistical differences were calculated with one-way ANOVA followed by Bonferroni post-test. For all statistical tests, *, **, ***, p < 0.05, 0.01, and 0.001, respectively.

Most mitochondria in untreated control cells were functional, and with treatment, the functional population decreased which corresponded to an increase in dysfunctional mitochondria (Fig. 18B, 18D). Interestingly, untreated VMP1 KO cells had similar populations of mitochondria to control cells although with LPS and ATP treatment, there was a drastic decrease in the percentage of functional mitochondrial and a large increase in the percentage of dysfunctional mitochondria (Fig. 18C, 18E). In LPS and ATP treated VMP1 KO cells, where instead of most mitochondria being functional, only about 20% of the mitochondria were functional (Fig. 18F). The inverse trend was observed for dysfunctional mitochondria where about 60% of the mitochondria were

dysfunctional in the LPS and ATP treated VMP1 KO cells compared to less than 20% in the other conditions (Fig. 18G).

Establishment of mitochondrial membrane potential is needed for normal cellular function, but under inflammatory conditions, it has been observed that there is a loss of membrane potential [217, 342]. To determine the relative membrane potential under these conditions, histograms of the max intensity of the CMXRos channel for each mitochondrion were generated (Fig. 18H). There was an increase in the percentage of mitochondria that have intermediate to low membrane potential in treated control cells. There was a dramatic loss in membrane potential in VMP1 KO cells treated with LPS and ATP such that at least 90% of the mitochondria have low membrane potential (Fig. 18I). Collectively, these data demonstrated that VMP1 KO cells have more and smaller mitochondria perhaps suggesting that there was more fission occurring. Additionally, treated VMP1 KO cells have a large population of dysfunctional mitochondria that corresponded to almost all the mitochondria having low membrane potential.

Treatment of control THP-1s with LPS and ATP resulted in an increase in the number of mitochondria that was comparable to VMP1 KO cells. The average number of mitochondria per cell was highest for VMP1 KO cells treated with LPS and ATP (Fig. 18J). These results were consistent with the average volume of mitochondria which showed that control THP-1s have the largest and most variability in volume. The other conditions had smaller average volumes (Fig. 18K). Previous work suggested that altered mitochondrial fusion can result in mitochondrial fragmentation and increased apoptotic cell death [210]. Our data supported that there may be a defect in

mitochondrial fusion in treated VMP1 KO cells given that there was an increase in the number of mitochondria as well as the presence of cleaved caspase-3 indicative of apoptotic cell death (Fig. 18J and 14G). Alternatively, it is known that when there is an accumulation of damage in mitochondria, fission separates damaged parts of the mitochondria for degradation [343]. Changes in mitochondrial fission and fusion under these conditions in VMP1 KO cells would need to be tested in future experiments.

VMP1 KO Cells have Increased Levels of Exposed Cytoplasmic mtDNA and Extracellular mtDNA.

Mitochondrial damage results in the release of mtDNA and mtROS [221, 222]. Since almost all the mitochondria in LPS and ATP treated VMP1 KO cells lost their membrane potential, it seems likely that there would be increased release of mtDNA from these cells. To determine whether there was increased exposed cytoplasmic mtDNA in VMP1 KO cells, differentiated THP-1s were treated then incubated with CMXRos and stained with anti-DNA antibodies (Fig. 19A and 19B). Only free cytoplasmic mtDNA is recognized by the anti-DNA antibody under the mild 0.1% saponin permeabilization conditions used in the following experiment. To quantify the number of DNA puncta inside and outside of the cell, cells were incubated with a fluorescent phalloidin probe to stain the actin filaments and identify the boundaries of the cell (Fig. 20A). In Imaris, an algorithm was built around the phalloidin stain (Fig. 20B). It was then possible to create two new channels: one that identified regions in an image that were inside of a cell and one that identified regions in an image that were outside of a cell. Voxels outside of the phalloidin surface algorithm were set to 0 to identify regions inside of the cell in a new channel (Fig. 20C). Voxels inside of the

phalloidin surface algorithm were set to 0 to identify regions outside of the cell in a new channel (Fig. 20D). In Imaris, a spots algorithm was then built around the DNA puncta. If the DNA puncta were inside of the cell, then they will be counted in the new channel that set the voxels outside of the phalloidin surface algorithm to 0. If the DNA puncta were outside of the cell, then they will be counted in the new channel that set the voxels inside of the phalloidin surface algorithm to 0.

Figure 19. Mild Detergent Conditions Allow for Detection of Exposed Cytoplasmic mtDNA. Differentiated WT THP-1s were fixed then permeabilized with either 0.1% or 1% saponin during incubation with an anti-DNA antibody. A) Schematic representing how saponin permeabilizes the plasma membrane but leaves the mitochondrial membrane intact. The anti-DNA antibody only binds to free cytoplasmic mtDNA under the 0.1% saponin permeabilization conditions used in the following experiments. B) Representative images of WT cells incubated with either 0.1% saponin or 1% saponin and the anti-DNA antibody.

Figure 20. Imaris Algorithm Built around a Phalloidin Stain Allows for Quantification of DNA Puncta Inside and Outside of the Cell. Differentiated control or VMP1 KO THP-1 cells were incubated with MitoTracker Red CMXRos dye to identify mitochondria, an anti-DNA antibody to detect mtDNA, and phalloidin which allowed for identification of the cell boundaries. A) Representative image of the anti-DNA puncta in green, the MitoTracker Red CMXRos in red, and the phalloidin staining in white. B) Imaris surface algorithm built around the phalloidin signal in red. C) A new channel was created by setting the voxels outside of the phalloidin surface algorithm to 0. Any puncta identified by the DNA spots algorithm in this new channel were considered inside of the cell. D) A new channel was created by setting the voxels inside of the phalloidin surface algorithm to 0. Any puncta identified by the DNA spots algorithm in this new channel were considered outside of the cell.

Representative images showed that only when VMP1 KO cells were treated with LPS and ATP was there an increase in DNA puncta (Fig. 21A). Imaris was utilized to build a surface around fluorescently labeled phalloidin which binds to actin in the cell. Then the number of DNA puncta inside and outside of the cell were quantified. The data demonstrated that there were relatively few DNA puncta inside of the control cells regardless of treatment, but there was a large increase in the number of DNA puncta inside of the cell in a field of view for LPS and ATP treated VMP1 KO cells (Fig. 21B). Interestingly, it was observed that there were DNA puncta on the coverslips outside of the cell in VMP1 KO cells treated with LPS and ATP (Fig. 21C). To quantify the cell-free mtDNA, qPCR was utilized where DNA was isolated from the supernatant and primers

specific to genes expressed in the small, circular mitochondrial genome were used.

Figure 21. LPS and ATP Treated VMP1 KO Cells have More Exposed Cytoplasmic and Released Extracellular mtDNA than Control Cells. VMP1 KO or control THP-1s were treated with LPS and ATP then analyzed as follows: A) Immunofluorescence microscopy images of cells stained with CMXRos dye and anti-DNA antibody. B) Quantification of the number of DNA+ puncta inside of the cell. C) Quantification of the number of DNA+ puncta outside of the cell. Data were quantified from 15 images per treatment group. D) qPCR data quantifying the number of mtDNA copies/µL in the supernatant. E) qPCR data representing the fold change of mtDNA/β2-microglobulin in the cell lysate. Data are representative of at least three independent experiments. Data are represented as mean ± SEM. Statistical differences were calculated using one-way ANOVA with Bonferroni post-test. For all statistical tests, *, **, ***, p < 0.05, 0.01, and 0.001, respectively.

Given that this is a sensitive assay, here mtDNA was detected in the supernatant of LPS and ATP treated control cells, but a larger number of mtDNA copies was detected in the supernatant from LPS and ATP treated VMP1 KO cells (Fig. 21D). These data were consistent with a recent study that quantified the release of mtDNA through GSDMD pores from BMDMs treated with LPS and nigericin, a pore-forming toxin that induces activation of the NLRP3 inflammasome [219]. Interestingly, there was no difference in the amount of mtDNA across conditions in the cell lysates even though there were more but smaller mitochondria in VMP1 KO cells suggesting that there was still the same amount of total mtDNA in these cells (Fig. 21E). Overall, these data suggested that VMP1 KO cells have more exposed cytoplasmic mtDNA that can be sensed and contribute to inflammatory responses as well as an increased release of mtDNA. Cell-free mitochondrial DNA (cfmtDNA) is increasingly being associated with pathogenic conditions including AD, PD, and MS [344].

VMP1 KO Cells have Disrupted Autophagic Flux and Increased Lysosomal Activity.

Since autophagy modulates inflammatory responses and previous reports suggested that VMP1 KO can impair autophagic flux, we wanted to assess autophagic processes in VMP1 KO macrophages under basal and inflammatory conditions [31, 222, 318]. To assess autophagosome/lysosome fusion, cells were stained for microtubule-associated protein 1A/1B-light chain 3 (LC3) and lysosome-associated membrane protein 1 (LAMP1). Double staining or colocalization of two structures suggested fusion of the autophagosome and lysosome (Fig. 22A). The number of LAMP1+ puncta were about the same between control and VMP1 KO cells with a slight

increase in control cells treated with LPS and ATP (Fig. 22B). The number of LC3+

puncta in VMP1 KO cells increased compared to control suggesting that there was an

accumulation in VMP1 KO cells (Fig. 22C). Interestingly, the number of LC3+ puncta

with LPS and ATP treatment for both control and VMP1 KO cells decreased, but in KO

cells it was the same average number as untreated control (Fig. 22C). In untreated

control cells, there was some colocalization between LC3 and LAMP1 suggesting fusion

of the autophagosome and lysosome which was disrupted in treated control cells and

untreated and treated VMP1 KO cells (Fig. 22D) which was consistent with previous

reports [5, 31]. Immunoblotting results demonstrated an increase in LC3II levels in

VMP1 KO cells and an accumulation of p62 compared to control suggesting that

autophagic flux was disrupted (Fig. 22E). Furthermore, to assess lysosomal function, a

Magic Red assay was used to measure cathepsin B activity, an enzyme involved in the

routine turnover of proteins. Bafilomycin A1 was used as a control because it raises

lysosomal pH resulting in the degradation of cathepsins. There was a slight increase in

cathepsin B activity in LPS and ATP control cells as well as about a two-fold increase in

untreated and treated VMP1 KO cells which was consistent with previous findings that

cathepsin B is required for NLRP3 assembly and activation (Fig. 22F) [345]. Taken

together, these data suggested that autophagy was disrupted in VMP1 KO cells and

that there was increased lysosomal activity which likely contributed to increased NLRP3

inflammasome activation and release of mtDNA.
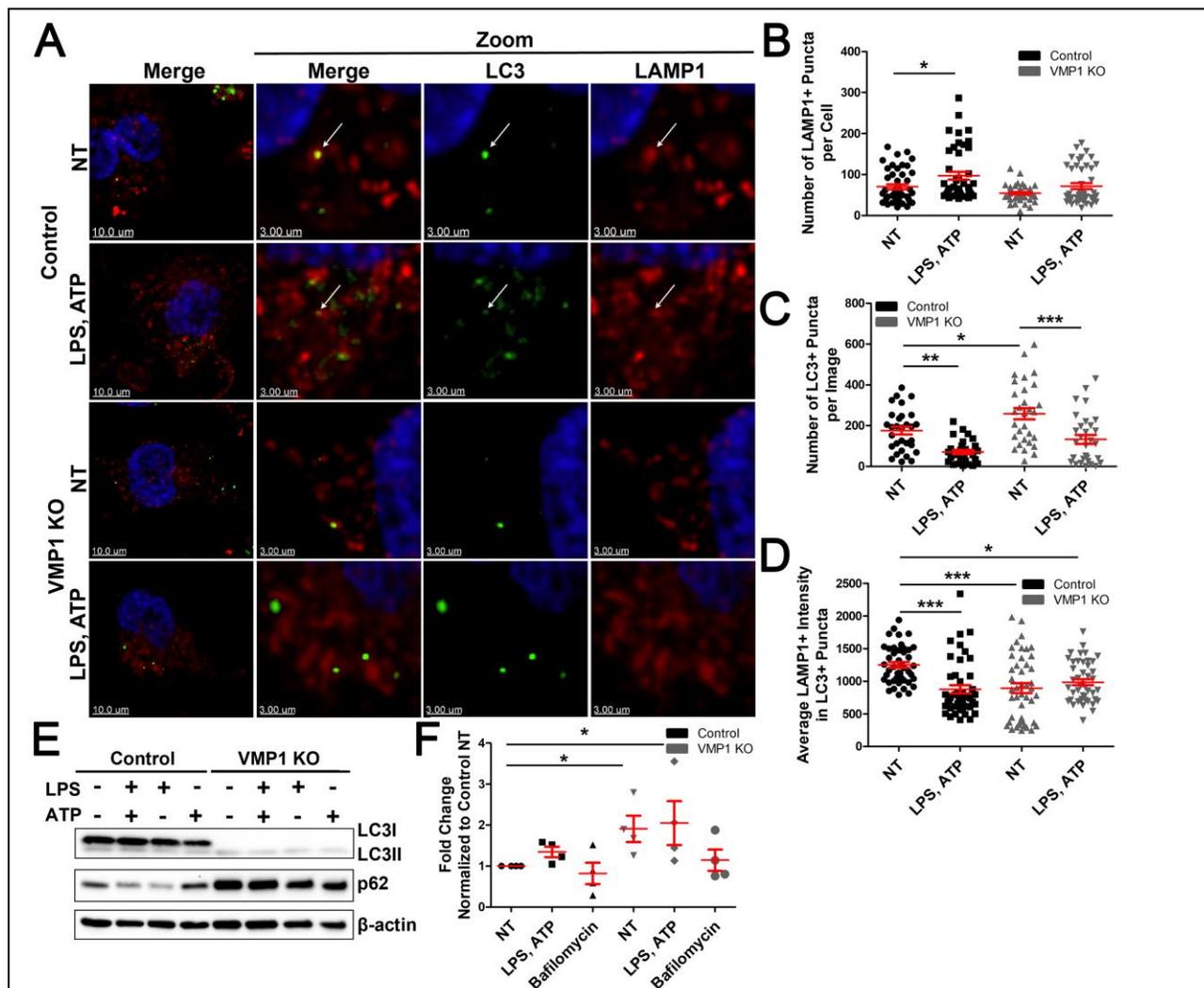
Figure 22. VMP1 KO Cells have Disrupted Autophagic Flux and Increased Cathepsin B Activity. A) Representative images from control or VMP1 KO cells untreated or treated with LPS and ATP then stained for LAMP1 and LC3. The white arrows point to colocalization of LAMP1 and LC3. B) Quantification of the number of LAMP1+ puncta per cell. C) Quantification of the number of LC3+ puncta per image. D) Quantification of the average LAMP1 intensity per image in LC3+ puncta. Data are from at least three independent experiments with at least 10 images per condition. E) LC3B and SQSTM1/p62 levels probed by western blot. F) Lysosomal dysfunction assay represented by the fold change in fluorescence of cells loaded with Magic Red dye indicative of cathepsin B activity. Statistical differences were calculated using one-way ANOVA with Bonferroni post-test or Dunnett's multiple comparison test. For all statistical tests, *, **, ***, p < 0.05, 0.01, and 0.001, respectively.

VMP1 KO Cells have Several Global Pathway Gene Expression Changes that Prime them for Proinflammatory Responses.

To determine global gene expression changes in VMP1 KO macrophages compared to CRISPR control cells, differentiated VMP1 KO or CRISPR control THP-1s were left untreated or treated with LPS and ATP. Each treatment condition was performed in triplicate. To confirm the phenotype of these samples, the supernatant was collected for a gal-3 ELISA (Fig. 23A). The data showed that there was an increase in the release of gal-3 from the VMP1 KO replicates treated with LPS and ATP compared to treated control cells. RNA was isolated and qPCR was performed to measure gene expression changes for proIL-1β, IL-6, and lgals3. Again, these data were consistent with previous experiments where LPS and ATP treated VMP1 KO THP-1s had an increased fold change in gene expression for proIL-1β and IL-6 compared to treated control cells (Fig. 23B and 23D). There was a slight increase in lgals3 gene expression in LPS and ATP treated VMP1 KO cells (Fig. 23C). These samples were then analyzed by RNA-Seq to identify global differential gene expression between conditions. A principle component analysis (PCA) plot was generated for the RNA-Seq data (Fig. 23E). Samples with similar expression profiles are clustered together. All three replicates of a given condition cluster together. Interestingly, there was no overlap between the different condition clusters. Principle component 1 reveals the most variation, and it separated the samples based on treatment vs. no treatment.

Figure 23. RNA-Seq Samples Demonstrate that VMP1 Negatively Regulates Gal-3 Release and Proinflammatory Cytokine Gene Expression. Control or VMP1 KO differentiated THP-1s were untreated or treated with LPS and ATP. A) Supernatant was collected and assessed by gal-3 ELISA. Cell lysates were collected following treatment with inflammatory stimuli and gene expression changes for B) proIL-1β, C) lgals3 and D) IL-6 were measured using qRT-PCR with gene specific primers. Graphs display fold change in mRNA gene expression compared with the NT control of either control or VMP1 KO THP-1s. E) PCA plot of the top 500 variable genes. Statistical differences were calculated using two-way ANOVA with Bonferroni post-test. For all statistical tests, *, **, ***, $p < 0.05$, 0.01, and 0.001, respectively.

Principle component 2 reveals the second most variation, and it separated the samples based on VMP1 KO vs. control. The data were then analyzed following a workflow that displayed upregulated and downregulated genes in a KEGG pathway [292].

Pathway analysis revealed that VMP1 KO cells were primed to recognize proinflammatory stimuli. Most toll-like receptors were upregulated in untreated VMP1 KO cells compared to control cells (Fig. 24A). This upregulation suggested that VMP1 KO cells were primed to recognize both extracellular and intracellular PAMPs. Analysis of TLR signaling pathways also identified upregulation of the transcription factors, NF-κB and AP-1, which are responsible for upregulating proinflammatory cytokine gene expression.

Figure 24. Heatmaps Representing Changes in Toll-like Receptor Signaling and Nod-like Receptor Signaling in VMP1 KO vs. Control Cells. Gene expression changes for untreated VMP1 KO cells compared to untreated control cells. A) Heatmap showing changes in gene expression for genes associated with Toll-like receptor signaling. B) Heatmap showing changes in gene expression for genes associated with Nod-like receptor signaling.

Additionally, expression of IKKβ, one of the kinases responsible for phosphorylating IκB subunits in the NF-κB:IκB complex triggering their ubiquitin-dependent degradation and activation of NF-κB, was also upregulated. In the steady state, it appeared that VMP1 KO cells were in a state primed for activation by PAMPs.

Several members of the Nod-like receptor signaling pathway as well as genes involved in inflammasome activation also had increased expression in VMP1 KO cells compared to control (Fig. 24B). ATP binds to and activates P2X7R to promote K^+ efflux and downstream inflammasome activation. Considering that P2X7R was upregulated in VMP1 KO cells, it suggested that KO cells were also primed to respond to DAMPs. Several sensors that ultimately oligomerize to form the inflammasome complex were also upregulated. These sensors included NLRP6, NOD1, NLRP3, NLRP12, NLRP1, and NLRC4 which are all activated by different stimuli. Although the focus of this study has been on the NLRP3 inflammasome activation, perhaps VMP1 KO cells may also experience exacerbated inflammatory responses when exposed to other inflammatory stimuli that activate some of these other sensors although this would need to be tested experimentally. Activation of the inflammasome results in activation of the protease, caspase-1 which was also upregulated in VMP1 KO cells compared to control. Upon activation, caspase-1 cleaves GSDMD and IL-18 which were both upregulated in untreated VMP1 KO cells. Collectively, analysis of genes involved in Toll-like receptor and Nod-like receptor signaling pathways suggested that VMP1 KO cells were primed to respond to inflammatory stimuli which corresponded to data from an earlier

experiment which showed an increased release of IL-1β from untreated VMP1 KO cells compared to control cells at a 24 h time point (Fig. 12C).

Analysis of genes involved in calcium signaling demonstrated that several calcium channels localized to the PM and the ER were upregulated (Fig. 25A). Experimental data showed that the calcium responses in VMP1 KO cells were dysregulated (Figs. 15 and 17). Interestingly, live cell imaging experiments showed that the Ca^{2+} ER stores were depleted in VMP1 KO cells (Fig. 15), and a previous study demonstrated that one function of VMP1 was to regulate SERCA activity [5]. This RNA-Seq data showed decreased expression of SERCA in VMP1 KO cells which suggested that perhaps reduced SERCA activity was due not only to the absence of VMP1 expression but also reduced expression of SERCA. The Na^+-Ca^{2+} exchanger (NCX) was upregulated in VMP1 KO cells. This channel is localized to the PM and the mitochondrial membrane. It typically transports Ca^{2+} out of mitochondria and outside of the cell while transporting Na^+ in the opposite direction. ORAI1, TRMPL, and RYR are calcium channels that were also upregulated in VMP1 KO cells. They all move Ca^{2+} into the cytoplasm. Experimental data measuring the intracellular $[Ca^{2+}]$ and Ca^{2+} levels in the mitochondria suggested that VMP1 KO cells have dysregulated Ca^{2+} responses (Figs. 15 and 17), yet how changes in the expression of these calcium channels might contribute to the dysregulation responses would need to be tested experimentally. Furthermore, several calcium/calmodulin-dependent protein kinases were also upregulated in VMP1 KO cells. These kinases are key regulators of calcium signaling and, in some cases, have been shown to mediate inflammatory responses [346].

Figure 25. Heatmaps Representing Changes in Calcium Signaling and the Cytosolic DNA-sensing Pathway in VMP1 KO vs. Control Cells. Gene expression changes for untreated VMP1 KO cells compared to untreated control cells in A and gene expression changes for LPS and ATP treated VMP1 KO cells compared to LPS and ATP treated control cells in B. A) Heatmap showing changes in gene expression for genes associated with calcium signaling. B) Heatmap showing changes in gene expression for genes associated with cytosolic DNA-sensing.

Overall, several Ca^{2+} channels were upregulated as well as other regulators of calcium signaling which was supportive of experimental data showing dysregulated calcium responses in VMP1 KO cells (Figs. 15 and 17). Additional experiments would need to be performed to determine whether changes in the expression of these calcium channels contribute to the responses observed in VMP1 KO cells.

VMP1 KO cells have increased exposed cytoplasmic levels of mtDNA which exacerbate inflammasome activation (Fig. 21). When comparing gene expression changes for the cytoplasmic DNA-sensing pathway for LPS and ATP treated VMP1 KO cells compared to treated control cells, the expression pattern suggested that VMP1 KO cells would respond more to exposed cytoplasmic mtDNA (Fig. 25B). Measurement of

inflammasome activation in VMP1 KO cells treated with the NLRP3 specific inhibitor, MCC950, showed that increased inflammasome activation in VMP1 KO cells was due to increased NLRP3 inflammasome activation (Fig. 14F). Several studies suggested the sensing of DNA by the cGAS/STING pathway resulted in NLRP3 inflammasome activation [235, 347]. In treated VMP1 KO cells, cGAS was upregulated with no change in STING expression which may increase sensing of exposed cytoplasmic DNA in these cells. Several other key inflammatory proteins were also upregulated in treated VMP1 KO cells versus treated control cells including NF-κB, GSDMD, procaspase-1, and AIM2 which can also sense DNA in the cytoplasm and form another inflammasome complex. Taken together, these data support the hypothesis that in VMP1 KO cells exposed cytoplasmic mtDNA is being sensed by the cGAS/STING pathway resulting in increased NLRP3 inflammasome activation although it would need to be tested by knocking out cGAS to see whether that would ameliorate exacerbated inflammatory responses.

Prior studies as well as our work demonstrated that autophagic flux was impaired in VMP1 KO cells [5, 31] (Fig. 22). Several genes involved in regulating autophagy were upregulated in VMP1 KO cells including SQSTM1 (p62) which accumulated in VMP1 KO cells as shown by western blot (Fig. 22E and 26A). Furthermore, there was increased expression of MAP1LC3B in VMP1 KO cells as well increased levels of LC3II by western blot (Fig. 22E and 26A). RNA-Seq data showed that there was increased expression of Akt, PTEN, and PI3K in VMP1 KO cells (Fig. 26A). These genes promote cell survival and perhaps activation of this pathway may balance dysregulated responses in VMP1 KO cells although this hypothesis would need to be tested.

Figure 26. Heatmaps Representing Changes in Genes Involved in Regulating Autophagy and Lysosome Associated Genes in VMP1 KO vs. Control Cells. Gene expression changes for untreated VMP1 KO cells compared to untreated control cells. A) Heatmap showing changes in gene expression for genes involved in regulating autophagy. B) Heatmap showing changes in gene expression for genes associated with lysosomes.

Furthermore, several genes associated with lysosomes were upregulated in VMP1 KO cells (Fig. 26B). MCOLN1 is a transmembrane protein that localizes to lysosomes and is involved in the late endosomal pathway and lysosomal exocytosis. Its activity is regulated by changes in $[Ca^{2+}]$ [348]. Perhaps upregulation of this gene is involved in the release of mtDNA from treated VMP1 KO cells although that would need to be tested. Several components of the V-ATPase were upregulated in VMP1 KO cells. V-ATPases are the proton pumps responsible for the acidification of lysosomes [349]. Further supporting increased lysosome activity in VMP1 KO cells was upregulation of a number of cathepsins which are lysosomal proteases that are involved in protein degradation, energy metabolism, and immune responses [350]. An assay using Magic Red which measures cathepsin B activity showed increased activity in untreated and

treated VMP1 KO cells compared to control cells (Fig. 22F). Several lysosome-associated genes were upregulated in VMP1 KO cells which along with the Magic Red data suggested that there was increased lysosomal activity in VMP1 KO cells. Collectively, the RNA-Seq data supported experimental data demonstrating that VMP1 negatively regulates inflammatory responses in macrophages and suggested several proteins that may be essential for the regulatory role of VMP1 in inflammatory responses that can be tested in future experiments.

CHAPTER FOUR

DISCUSSION

In this study, we investigated how the protein, VMP1 known for its ability to modulate SERCA activity and autophagy, may regulate inflammatory responses (Fig. 27). It is increasingly appreciated that disruption of autophagy results in dysregulated inflammatory responses that are often associated with disease [351]. We used CRISPR-Cas9 to genetically delete VMP1 to elucidate whether VMP1 regulates innate inflammatory responses [5]. This study is the first to characterize the role of VMP1 in innate immune responses and autophagic processes in macrophages. Initial experiments demonstrated that VMP1 negatively, yet differentially regulated the release of IL-1β, IL-6, and gal-3 in response to multiple NLRP3 inflammasome agonists (Fig. 12). Perhaps this differential secretion depending on the agonist was due in part to different mechanisms of secretion for each of these proteins. For example, our work demonstrated that gal-3 can be released in EVs, but that it was predominantly released through GSDMD pores in response to inflammatory stimuli in differentiated THP-1s (Fig. 11). Similarly, IL-1β typically is thought of for its secretion through GSDMD pores in response to inflammatory stimuli. Yet the literature demonstrates that this is not the only pathway of secretion for IL-1β.

Figure 27. Working Model for how VMP1 Regulates Inflammatory Responses.
Figure legend continued on the next page.
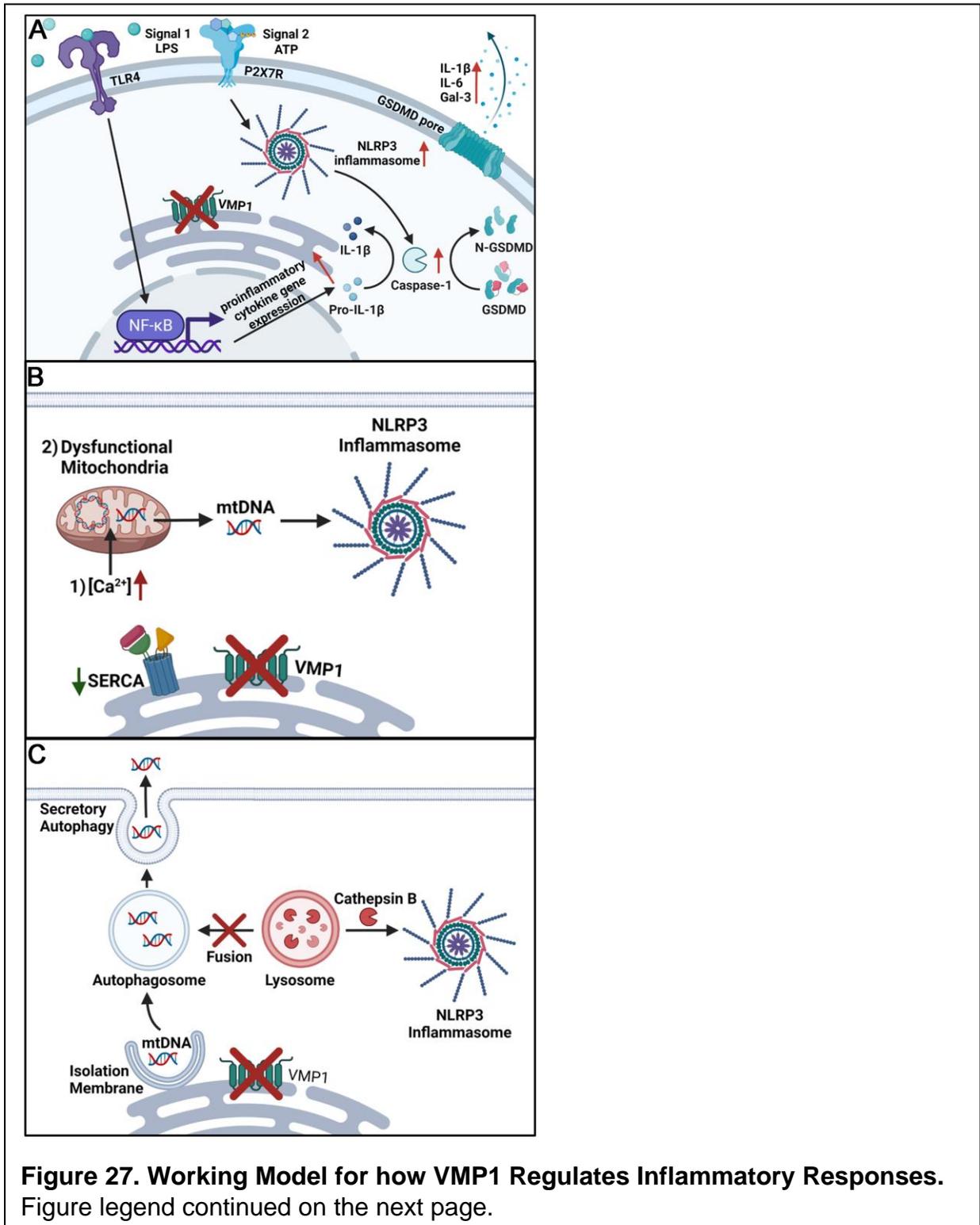
Figure 27. Working Model for how VMP1 Regulates Inflammatory Responses. A) Overview of how VMP1 regulates inflammatory pathways: In VMP1 KO cells treated with LPS and ATP, there was increased proinflammatory cytokine gene expression, NLRP3 inflammasome activation, caspase-1 activation, and release of IL-1β, IL-6, and gal-3. These exacerbated inflammatory phenotypes in VMP1 KO cells were likely due to the two known functions of VMP1: regulation of SERCA activity and autophagy. **B) SERCA activity:** VMP1 KO cells have decreased expression of SERCA accompanied by decreased SERCA activity. Upon activation with ATP, VMP1 KO cells have elevated cytoplasmic $[Ca^{2+}]$. Mitochondria in VMP1 KO cells have increased influx and decreased efflux of Ca^{2+} resulting in loss of membrane potential, mitochondrial dysfunction, and exposure of cytoplasmic mtDNA. mtDNA is then hypothesized to act as a DAMP to increase activation of the NLRP3 inflammasome in VMP1 KO cells. **C) Autophagy:** VMP1 KO cells exhibited impaired autophagic flux specifically at the stage of autophagosome/lysosome fusion. In VMP1 KO cells, there was tethering of the isolation membrane with the ER. However, some autophagosomes still formed and we hypothesize that the extracellular mtDNA we detect is being released through secretory autophagy. Additionally, VMP1 KO cells have increased cathepsin B activity which we hypothesize also contributes to increased NLRP3 inflammasome activation.

When IL-1β is cleaved, it becomes positively charged which facilitates its movement the plasma membrane where its fast release is mediated through GSDMD pores [352] and its slow release is mediated through a GSDMD-independent pathway that has yet to be fully elucidated [353]. Additionally, IL-6 release occurs through the conventional ER-Golgi mechanism of secretion [354]. Evidence in the literature suggests that VMP1 can regulate protein secretion [16]. Thus, it would be interesting in future studies to elucidate which secretory pathways these proinflammatory molecules are utilizing in VMP1 KO cells because it seems feasible to hypothesize that there would be differences between cells in which VMP1 was knocked out compared to cells expressing normal levels of VMP1.

Increased release of IL-1β in response to the canonical NLRP3 inflammasome activators, LPS and ATP, in VMP1 KO cells suggested this phenotype was due in part to increased inflammasome activation. Interestingly, even though LPS and poly(I:C) treatment alone upregulated proIL-1β expression to the same extent as LPS and ATP treatment, there was no difference in IL-1β release from VMP1 KO compared to control. Similar secretion patterns were observed for IL-6 and gal-3 in response to inflammatory stimuli in VMP1 KO compared to control which differed from the secretion patterns observed from cells depleted of other autophagy-associated proteins. [355, 356]. Given that there are multiple mechanisms for the secretion of IL-1β, IL-6, and gal-3 with some overlap, it seems plausible that VMP1 regulates secretory pathways although this idea would need to be tested in future studies [134, 357-359].

VMP1 KO macrophages had increased caspase-1 activation in response to LPS and ATP (Fig. 14). Use of MCC950, a specific inhibitor of the NLRP3 inflammasome, indicated that this increase in caspase-1 activation was mediated specifically by the NLRP3 inflammasome. Many agonists can activate the NLRP3 inflammasome by inducing a variety of downstream signals, yet a consensus model for activation has not yet been identified [360]. Data acquired in VMP1 KO cells suggested that multiple signals associated with NLRP3 inflammasome activation were present when cells were activated by canonical inflammatory stimuli. These signals included Ca^{2+} mobilization, mitochondrial dysfunction, and release of mtDNA (Fig. 15, 17, 18, and 21). Although Ca^{2+} mobilization as a signal upstream of NLRP3 inflammasome activation is controversial, our data demonstrated that in control THP-1s treated with thapsigargin,

an inhibitor of the Ca^{2+} ATPase SERCA, alongside LPS and ATP, there was an increase in caspase-1 activation like what was observed in VMP1 KO cells that have diminished SERCA activity (Fig. 15) [5, 188, 189, 202]. Our data suggested that Ca^{2+} mobilization alone was not sufficient to induce inflammasome activation (Fig. 16), but impaired responses to changes in cytoplasmic $[Ca^{2+}]$ exacerbated responses to additional inflammatory stimuli. Measurement of changes in $[Ca^{2+}]$ in proximity to mitochondria in response to ATP showed that there was an increased rate of influx and a decreased rate of efflux in VMP1 KO cells compared to control (Fig. 17). Based on what is known in the literature, we believe that Ca^{2+} overload in the mitochondria resulted in the loss of mitochondrial membrane potential and release of mtDNA (Fig. 18 and 21). Extension of MAMs as is the case for VMP1 KO cells affects the transfer of Ca^{2+} from the ER to mitochondria and in our case resulted in a mitochondrial Ca^{2+} overload [5, 361]. Dysregulated opening of the mPTP triggers the release of matrix metabolites leading to the loss of mitochondrial membrane potential, inhibition of oxidative phosphorylation, and mitochondrial swelling [362-364]. Furthermore, in VMP1 KO cells we observed increased levels of cleaved caspase-3 when cells were treated with inflammatory stimuli, and previous work has shown that NLRP3 activators can trigger apoptosis leading to the loss of mitochondrial membrane potential and release of mtDNA into the cytosol which can then trigger NLRP3 inflammasome activation [221]. Overall, we hypothesize that in VMP1 KO cells treated with inflammatory stimuli, there is an increase in $[Ca^{2+}]$ that results in the loss of mitochondrial membrane potential and

release of mtDNA which we believe is being sensed by the cGAS/STING pathway to increase NLRP3 inflammasome activation.

This work introduces a novel approach to assess mitochondrial function in cells. Live cell confocal microscopy allowed us to visualize individual mitochondria in cells which was an improvement on the resolution of the previous assay. Analysis of these images using Imaris allowed for an unbiased approach where an algorithm was built around individual mitochondria that were identified by MitoTracker Green dye which accumulates in mitochondria based on their mass but independent of membrane potential. Using this approach, we can determine several parameters of the individual mitochondria including the number, size, and volume which can give a sense for how mitochondrial dynamics change in cells under different conditions which is information that is lost in the flow cytometry-based assay. Furthermore, it allowed for the mitochondrial membrane potential to be quantified in each mitochondrion. The data can be displayed in plots analogous to flow plots to allow for quantification of the relative percentages of mitochondria that can be considered functional or dysfunctional, yet there was improved resolution in our assay where we were able to determine the relative percentage of mitochondria that have low, intermediate, or high mitochondrial membrane potential. This work established a robust assay for characterizing the mitochondrial dynamics in untreated or treated cells.

Our work identified that VMP1 KO cells have an increased release of mtDNA that was quantified in the supernatant by qPCR using primers specific to mitochondrial genes, but we also utilized a microscopy-based approach to quantify the release of

mtDNA. We used mild saponin conditions that allowed for us to only detect exposed

cytoplasmic mtDNA (Fig. 19), and we stained the cells with phalloidin to leverage Imaris

to build an algorithm around the phalloidin stain such that we could identify mtDNA

puncta that were located inside or outside of the cell (Fig. 20). Future studies can utilize

this microscopy-based approach to determine how mtDNA is being released from VMP1

KO cells. We hypothesize that it is through secretory autophagy because of decreased

autophagosome/lysosome fusion in VMP1 KO cells, but this would need to be tested

directly. Furthermore, this imaging-based approach would address the question of

whether dysfunctional mitochondria are also being released from the cell along with the

mtDNA.

Previous studies have demonstrated that in VMP1 KO cells autophagy is

impaired at autophagosome/lysosome fusion [5, 31]. Our data was consistent with these

findings with our data showing that in VMP1 KO cells there was less colocalization

between LC3 and LAMP1 and an accumulation of p62 (Fig. 22). We hypothesize that

this defect in autophagy likely resulted in the persistence of dysfunctional mitochondria

and increased NLRP3 inflammasome activation in VMP1 KO cells [217, 221, 222, 365].

Along these lines, previous work demonstrated that activation of the AIM2 and NLRP3

inflammasomes trigger caspase-1 dependent mitochondrial damage. This mitochondrial

damage includes mtROS production, loss of mitochondrial membrane potential, and

inhibition of mitophagy which likely amplifies inflammatory responses due to

mitochondrial damage [366]. Interestingly and perhaps in contrast to this previous study,

in our study we observed a modest increase in mitochondria that lost their membrane

potential with LPS and ATP activation in control cells compared to almost all

mitochondria losing their membrane potential in VMP1 KO cells (Fig. 18). Perhaps

because our experiments were performed 30 min after adding ATP, the phenotype we

observed for VMP1 KO cells was more pronounced compared to control cells. Maybe

we would observe more evidence of mitochondrial damage at later time points following

caspase-1 activation in control cells. Overall, it seems likely that impaired autophagic

flux in VMP1 KO cells would allow for damaged mitochondria to persist and augment

inflammatory responses.

Typically, with NLRP3 activation, p62 is recruited to damaged mitochondria

which are then ubiquitinated through a Parkin-mediated mechanism for degradation, but

in VMP1 KO cells mitophagy is defective and there is an accumulation of damaged

mitochondria [150, 342]. Additionally, upon inflammasome activation, p62 interacts with

ASC targeting the inflammasome components to autophagosomes for degradation to

control inflammatory responses which is another mechanism that is likely defective in

VMP1 KO cells [318]. Aside from promoting inflammasome activation, autophagy

inhibition can elevate IL-1β release due to an increase in available proIL-1β for cleavage

in the cytosol similar to what we observed in VMP1 KO cells (Fig.12) [313]. Cathepsin B

which is released from damaged lysosomes was more active in VMP1 KO cells and has

been shown to be essential for NLRP3 inflammasome activation (Fig. 22) [345, 367].

Our work demonstrated that there was increased release of mtDNA from VMP1 KO

cells in response to LPS and ATP compared to control cells (Fig. 21). Recent work

suggested that mtDNA is primarily released from the cell due to membrane rupture, but

a fraction of the mtDNA can also be released through GSDMD/gasdermin E (GSDME) pores upon pyroptotic/apoptotic cell death [219]. We hypothesize that it is more likely that in VMP1 KO cells due to diminished autophagosome fusion with the lysosome, there would be increased fusion of the autophagosome with the plasma membrane to release mtDNA through secretory autophagy [368].

Even though this work primarily focused on addressing how two known functions of VMP1 particularly its modulation of SERCA activity and autophagic flux may be mediating exacerbated inflammatory responses, there are additional functions of VMP1 that may be contributing to this phenotype. For example, it is known that in VMP1 KO cells there is this sustained interaction between the ER membrane and several organelles that is mediated by the interaction between VMP1 and SERCA [5]. These sustained membrane contacts may also influence inflammasome activation, and it would be interesting to look at the localization of inflammasome components in VMP1 KO cells before and after treatment with inflammatory stimuli. In an unstimulated cell, NLRP3 localizes to the ER and cytosol, but upon stimulation, NLRP3 in the ER localizes adjacent to ASC in mitochondria [369, 370]. The NLRP3 inflammasome complex is assembled at highly specialized contact sites between the ER and mitochondria known as MAMs [369]. It is thought that this localization of NLRP3 to MAMs/mitochondria may contribute to the immediate recognition of and response to mitochondrial damage and mtDNA translocation [369]. Perhaps the increased presence of MAMs in VMP1 KO cells may be the reason for increased basal inflammatory responses compared to control cells. Maybe the proximity of NLRP3 and ASC in VMP1 KO cells may result in the

formation of an inflammasome complex under basal conditions that can then cleave

proIL-1β and allow for it to be released from the cell (Fig. 12C). Even though the priming

signal typically results in the upregulation of inflammasome components and

proinflammatory cytokine gene expression, the basal level of proIL-1β is high in

differentiated THP-1 cells suggesting that there may be sufficient levels for it to be

cleaved in untreated VMP1 KO cells.

As mentioned previously, VMP1 is an integral membrane protein localized to the

ER, but recently, it has been identified to also have phospholipid scramblase activity

which is important for maintaining the normal distribution of phosphatidylserine and

cholesterol [14, 371]. In the absence of VMP1, more cholesterol becomes accessible on

the cytoplasmic leaflet of the PM as well as an increase in phosphatidylserine in the

cytoplasmic leaflet of cellular membranes. It is thought that the role of VMP1 in

maintaining cellular lipid distribution may explain how VMP1 can be involved in so many

different cellular processes. Lipid composition in the ER can greatly impact the

biogenesis of autophagosomes suggesting another way for how VMP1 can be

influencing autophagy and inflammatory responses in macrophages independently of its

ability to alter $[Ca^{2+}]$ [372, 373]. Furthermore, elevated levels of cholesterol can also

increase inflammatory responses. For example, excess cholesterol can form cholesterol

crystals which can directly activate the NLRP3 inflammasome [374]. Non-crystalline

cholesterol at high concentrations also can trigger inflammasome activation by impairing

mitochondrial function [375, 376]. Yet other studies suggest that cholesterol can have

inhibitory effects on the activation of NLRP3 inflammasome activation so the role of

cholesterol in inflammasome activation is still unclear [377]. Whether the scramblase

activity of VMP1 is involved in exacerbated inflammatory responses is unknown, but it

seems possible that it may be involved although perhaps indirectly.

Our data demonstrated that there was increased mitochondrial dysfunction and

release of mtDNA in VMP1 KO cells (Fig. 18 and 21), yet we have not measured the

levels of mtROS which are likely elevated in VMP1 KO cells. Following mitochondrial

damage mtROS can be produced and oxidize mtDNA to activate the NLRP3

inflammasome [221]. It has been shown that ATP can induce the production of mtROS

to activate the inflammasome [378]. Yet several studies have found that mtROS is

dispensable for NLRP3 inflammasome activation where serum β-amyloid can induce

activation through mtROS-dependent and mtROS-independent mechanisms [379]. In

contrast, some viruses can activate the inflammasome in a mtROS-independent

manner [227, 380]. Overall, it remains controversial whether mtROS is essential for

NLRP3 inflammasome activation. Another source of signals that activate the

inflammasome and may be affected in VMP1 KO cells are derived from lysosomal

permeabilization. LPS and ATP induce lysosomal destabilization, and cathepsin B, a

lysosomal cysteine protease, has been found to be required for caspase-1 activation,

ASC speck formation, and IL-1β production [345]. In VMP1 KO cells, there was

increased cathepsin B activity in untreated cells that was increased compared to control

untreated cells (Fig. 22). Perhaps the increased levels of cathepsin B in VMP1 KO cells

may contribute to increased basal inflammatory responses in VMP1 KO cells. It would

be interesting to further characterize the function of the lysosome in VMP1 KO cells and

more specifically elucidate whether it contributes to elevated inflammatory responses in VMP1 KO cells. Given that several signals that can induce NLRP3 inflammasome activation are elevated in VMP1 KO cells, but not all of them are required, it would be interesting to tease apart which ones are required for increased inflammasome activation in these cells. It can sometimes be difficult to inhibit these signals directly without off target effects, but it would help to define the mechanism for how the host protein, VMP1, negatively regulates inflammatory responses.

In summary, we showed that in VMP1 KO cells in response to inflammatory stimuli there was increased release of IL-1β, IL-6, and gal-3. In VMP1 KO cells, there was increased NLRP3 inflammasome and caspase-1 activation. Due to inhibition of SERCA activity in VMP1 KO cells, there were elevated levels of cytoplasmic $[Ca^{2+}]$ and increased influx and decreased efflux rates of Ca^{2+} from mitochondria upon ATP stimulation which caused a loss of membrane potential in almost all mitochondria and increased the release of mtDNA. VMP1 KO impaired autophagic flux at the stage of autophagosome/lysosome fusion which likely prevented the cells from degrading damaged mitochondria further exacerbating inflammatory responses. Taken together, our findings identify a novel negative regulatory role for VMP1 in inflammatory responses. VMP1 restricts NLRP3 inflammasome activation through its modulation of SERCA activity and autophagy. In the broader context, decreased expression of VMP1 has been identified in the monocytes of patients with Primary-Progressive MS and in the PBMCs of PD patients [40, 90]. The progression of these neurologic diseases is characterized by dysregulated inflammatory responses that contribute to devastating

disease sequalae. Our findings support a potentially critical role for VMP1 in the progression of inflammatory responses in these diseases that perhaps can one day be targeted therapeutically.

Concluding Remarks.

In this work, for the first time, the function of VMP1 was characterized in macrophages specifically how it can regulate inflammatory responses was outlined. Depletion of VMP1 resulted in increased basal release of IL-1β along with elevated levels of proinflammatory cytokines and gal-3 being released in response to proinflammatory stimuli. In part, this exacerbated inflammatory response was mediated by increased NLRP3 inflammasome activation. In connection with disease, VMP1 KO cells treated with LPS and α-syn fibrils released more IL-1β compared to control cells suggesting that PD patients with decreased expression of VMP1 would have increased inflammatory responses with exposure to inflammatory stimuli but also due to α-syn fibrils characteristic of disease pathology. It would be interesting to determine if there are other diseases associated with decreased VMP1 expression and to further characterize the function of VMP1 in additional immune cell types both in the periphery and the CNS.

This work demonstrated that in VMP1 KO macrophages there was decreased SERCA activity and impaired autophagic flux consistent with what was observed previously [5, 31]. Regulation of $[Ca^{2+}]$ and autophagy both likely contributed to increased inflammasome activation. The data showed that elevated $[Ca^{2+}]$ in response to ATP increased $[Ca^{2+}]$ in the mitochondria which led to loss of membrane potential

and the release of mtDNA. Additionally, impaired autophagy would prevent the degradation of damaged mitochondria as well as the removal of activated inflammasome components which likely further exacerbate inflammatory responses over the long-term. Further studies will need to be done to address to what extent each function of VMP1 contributes to regulating inflammatory responses especially given the pleiotropic role of VMP1. With this work as the foundation, many questions remain about how VMP1 may be regulating cellular responses in immune cells. Future work needs to be done to characterize the mechanisms underlying how VMP1 regulates inflammatory responses broadly but also more specifically in the context of PPMS and PD. Ultimately, it would be useful for a therapeutic to be developed that could fine-tune the expression of VMP1 in different disease states with it potentially being able to mitigate excessive and unwanted inflammatory responses that typically contribute to disease progression in PPMS and PD until cures for these diseases can be developed.

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VITA

Stephanie Zack was born in Hinsdale, Illinois, on June 12, 1994 to Steven and Linda Zack. She earned her Bachelor of Arts degree in Chemistry from Carthage College in Kenosha, Wisconsin, in May 2016. During her undergraduate studies, Stephanie worked in the laboratory of Dr. Kevin Morris, studying chiral recognition by molecular micelles with molecular dynamics simulations.

After graduation, Stephanie then matriculated into the Loyola University Chicago Stritch School Infectious Disease and Immunology Graduate Program under the mentorship of Dr. Edward Campbell. Stephanie's thesis work focused on understanding how lidocaine and Zep peptides attenuate inflammatory responses. In 2018, Stephanie matriculated into the Loyola University Chicago Interdisciplinary Program in Biomedical Sciences. She joined the Department of Microbiology and Immunology and continued under the mentorship of Dr. Edward Campbell studying how the protein, vacuole membrane protein 1 (VMP1), regulates inflammatory responses. This work was supported by the T32 Immunology Training Grant awarded to Dr. Katherine Knight.

After completion of her graduate studies, Stephanie will continue to pursue her interest in scientific research and begin a postdoctoral position at the University of Illinois Chicago in the laboratory of Dr. Shiva Shahrara. She will investigate Notch signaling in rheumatoid arthritis.