

**DISSECTING THE MECHANISM OF NLRP3 INFLAMMASOME ACTIVATION IN  
INDIVIDUAL CELLS: THE ROLE OF REACTIVE OXYGEN SPECIES AND  
ORGANELLE DAMAGE**

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# **DISSECTING THE MECHANISM OF NLRP3 INFLAMMASOME ACTIVATION IN INDIVIDUAL CELLS: THE ROLE OF REACTIVE OXYGEN SPECIES AND ORGANELLE DAMAGE**

Michelle Elizabeth Heid, Ph.D.

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Inflammation underlies the pathology of numerous diseases. It can be initiated by macrophages through the secretion of pro-inflammatory cytokines, such as IL-18 and IL-1 $\beta$ , following the activation of a molecular complex called the inflammasome. Inflammasomes are a protein scaffolding complex consisting of three known components: a sensory NLR, such as NLRP3, ASC, and caspase-1. The NLRP3 inflammasome is activated by a diverse array of stimuli, including crystals, ATP, pore-forming toxins, such as tetanolysin O (TLO), and the potassium ionophore nigericin. Here, we have explored the mechanism of NLRP3 inflammasome activation using nigericin, TLO and ATP. We found that nigericin induced NLRP3 inflammasome activation in bone-marrow derived macrophages (BMDM) results in inflammasome dependent lysosomal membrane permeabilization (LMP), mitochondrial membrane permeabilization (MMP), and the processing and secretion of IL-1 $\beta$ . All of these events required mitochondrial reactive oxygen species (ROS). Through combining bulk biochemical assays with live cell analysis of individual cells, we provide a kinetic analysis and sequence of events resulting from nigericin stimulation of LPS primed BMDM. We recapitulated NLRP3 inflammasome activation in a dendritic cell line, using the novel system of D2SC-1 transduced with ASC. These transduced cells undergo a similar sequence of events as macrophages, confirming that this mechanism is a general result of NLRP3 inflammasome activation applicable to multiple cell types. Furthermore, we have found that ATP and TLO stimulation of BMDM results in a similar

sequence of NLRP3 dependent events. ATP, but not nigericin, requires P2X7 for activation of the NLRP3 inflammasome. Surprisingly, inhibition of P2X4 blocked nigericin, but not ATP, induced, NLRP3-dependent IL-1 $\beta$  secretion in BMDM. Our work has demonstrated a central, common role for mitochondrial ROS in NLRP3 inflammasome activation and determined the kinetics of organelle crosstalk during inflammasome activation. These data place both the mitochondria and lysosomes in a critical position controlling NLRP3 inflammasome activation. Based on these results, we suggest mitochondrial ROS as a potential therapeutic target for treating NLRP3 inflammasome related diseases. Inhibition or scavenging of mitochondrial ROS would not only prevent the pro-inflammatory effects of IL-1 $\beta$  secretion in these patients, but also NLRP3 dependent organelle damage and the resulting cell death.

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## PREFACE

Attending graduate school at the University of Pittsburgh has been a rich and rewarding experience. I deeply value the education and training I have received during my time here. Additionally, I have met numerous people who have helped shape my life and career in positive ways. First, I would like to thank my mentor, Dr. Russell Salter, for providing guidance and encouragement throughout my time as a graduate student. His advice and patience has been invaluable to both the development of my project and helping me work toward future career goals. I would also like to thank the past members of the Salter lab, including Jessica Chu, L. Michael Thomas, Peter Keyel, Chengqun Sun, and Sarita Singh for their assistance in research endeavors as well as for creating a positive lab environment.

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## 1.0 INTRODUCTION

One crucial process involved with successful resolution of pathogenic conditions is inflammation. Originally characterized in the 1<sup>st</sup> century AD by Celsus by four clear signs: *rubor*, *tumor*, *calore* and *dolore* (redness, swelling, heat and pain) (Rocha e Silva, 1994), inflammation was later expanded to also include loss of function. Although there has been debate through the centuries whether inflammation was primarily beneficial or harmful, it is now known that it depends on the specific circumstances of inflammation. For vaccine development and the clearance of most pathogens, inflammation is necessary to develop the needed immune response. However, excessive inflammatory responses to certain pathogens cause extensive tissue damage, and inflammatory responses to non-pathogenic conditions results in autoimmunity. Understanding how to balance inflammation between the amount necessary to clear pathogens, without causing extensive tissue damage, is central to successfully treating many diseases, including cancer, diabetes, atherosclerosis, rheumatoid arthritis, and lupus.

Inflammation is initiated by the innate immune system. The innate immune system comprises cells that are designed to immediately react to stimuli that are potentially pathogenic—such as pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Upon recognition of these PAMPs and DAMPs, innate immune cells attempt to resolve the pathogenic condition through a variety of means, as well as signal to

the rest of the immune system via antigen presentation to solicit assistance in clearing the problem. There are a wide variety of innate immune cells that were initially characterized either morphologically or by function. These cells include basophils, eosinophils, neutrophils, mast cells, dendritic cells and macrophages. Each cell type plays an important and likely unique role in a variety of immune responses. Macrophages represent one of the key regulators of inflammation. In this chapter I will focus on the role of myeloid cells, especially macrophages and dendritic cells, in innate immunity and inflammation. More specifically, I will examine a unique complex, the inflammasome, which is present in a variety of immune cells, and its role in inflammation.

## **1.1 MACROPHAGES**

Macrophages were originally identified in 1905 by their ability to phagocytose bacteria and their apparent resistance to pathogens (Martinez et al., 2009). They are important antigen presenting cells that originate from the bone marrow and then travel to the tissue (Shaughnessy and Swanson, 2007). In addition to regulating inflammation in the context of both the innate and the adaptive immune responses, macrophages also influence inflammation through the regulation of lipid metabolism, iron metabolism, and wound repair (Classen et al., 2009). As innate immune cells, macrophages play a key role in the removal of bacterial pathogens through phagocytosis. Once phagocytosed, bacteria are destroyed by macrophages in a contained environment to avoid harming surrounding tissue (Ernst, 2000). Macrophages destroy bacterial pathogens through the production of reactive nitrogen and oxygen species (RNOS) that are contained within phagosomes. Inducible nitric oxide synthase (iNOS)



produces nitric oxide (NO). The NADPH oxidase complex produces superoxide in the lumen of the phagosome, where it is then used to produce other reactive oxygen intermediates via luminal superoxide dismutase (Shaughnessy and Swanson, 2007). NO, superoxide, and some of their products, such as peroxynitrite, are bactericidal (Shaughnessy and Swanson, 2007). Macrophages also restrict the availability of iron and tryptophan in the phagosome, which can prevent bacterial growth (Mosser, 2003). Thus, macrophages utilize a variety of methods to directly eliminate pathogens.

Along with destroying bacterial pathogens, macrophages also signal to the rest of the immune system through the production of cytokines. When macrophages are exposed to the pro-inflammatory cytokines Interferon  $\gamma$  (IFN $\gamma$ ) and tumor-necrosis factor  $\alpha$  (TNF $\alpha$ ), or a PAMP such as lipopolysaccharide (LPS), they become classically activated (Mosser, 2003). Macrophages activated in this fashion secrete the pro-inflammatory cytokines TNF $\alpha$ , Interleukin (IL)-12, IL-1 $\beta$ , and IL-6, increase surface expression of MHC class II and CD86 molecules, and decrease mannose receptor expression (Mosser, 2003). Classically activated macrophages produce RNOS and can help induce a Th1 cellular immune response (Mosser, 2003). Classical activation of macrophages also increases their ability to phagocytose and destroy bacterial pathogens (Mosser, 2003). On the other hand, exposure of macrophages to IL-4, IL-13, IL-10, or glucocorticoids produces alternatively activated macrophages (Classen et al., 2009). Unlike classically activated macrophages, alternatively activated macrophages do not produce NO and are not thought to have an enhanced ability to kill bacteria (Mosser, 2003). Instead, alternatively activated macrophages are involved with the reorganization of the extracellular matrix and wound repair (Mosser, 2003). Alternatively activated macrophages secrete the anti-inflammatory cytokine IL-10 and have increased surface expression of mannose receptor and CD23 (Mosser, 2003). Furthermore, alternatively activated macrophages downregulate pro-inflammatory

responses including caspase-1 activity and IL-1 $\beta$  production (Martinez et al., 2009). Thus, macrophages can promote or inhibit inflammation, depending on signals they receive from their environment.

## 1.2 PATTERN RECOGNITION RECEPTORS

The signals that lead macrophages to initiate a pro-inflammatory response have been partially elucidated. Primarily, these comprise the PAMPs and DAMPs, which are sensed on the macrophage primarily via two families of pattern recognition receptors (PRRs). Toll-like receptors (TLRs) were the first described family of PRRs in mammals, which are homologs to *Drosophila* Toll (Lemaitre et al., 1996; Medzhitov et al., 1997; Nomura et al., 1994; Taguchi et al., 1996). Similar to Toll, activation of TLRs induces a robust immune response following ligation (Lemaitre et al., 1996; Medzhitov et al., 1997). The prototypical TLR, TLR4, responds specifically to lipopolysaccharide present in the cell wall of Gram negative bacteria (Poltorak et al., 1998). Similarly, other TLRs specifically respond to other molecules that are not present in mammalian cells, including bacterial or viral DNA, bacterial proteoglycans, and flagellin (O'Neill et al., 2013). TLRs recognize these PAMPs through a leucine-rich repeat region (LRR) that either directly bind to the PAMP, or to other proteins that directly bind to the PAMP (Bell et al., 2005; Miyake, 2003). TLR ligation can induce pro-inflammatory transcriptional changes through the transcription factor NF- $\kappa$ B and AP-1 and induction of Type I interferons (O'Neill et al., 2013).

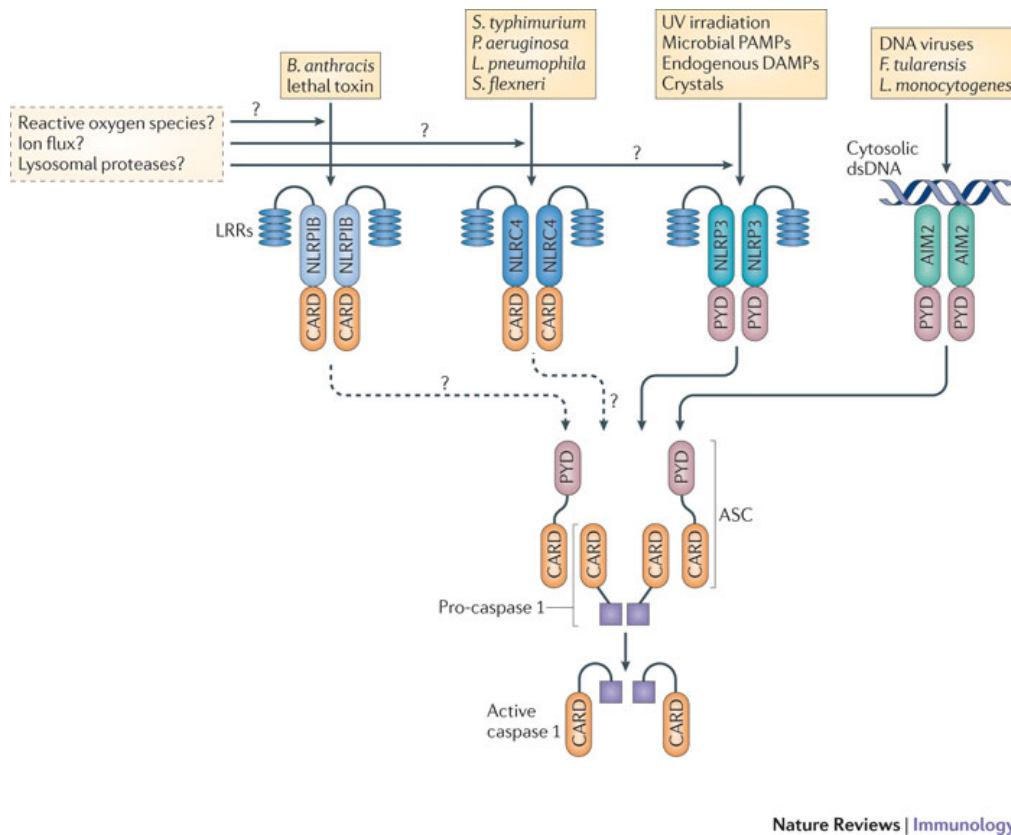
LPS binds to TLR4 via the LPS binding protein and CD14 (Bode et al., 2012). Following oligomerization of TLR4 and signaling through the TIR domain, adaptor proteins, such as MyD88, TRIF, and TRAM are recruited. MyD88 can activate p38<sup>MAPK</sup> and eventually lead to IκB releasing NFκB and gene transcription (Bode et al., 2012). Therefore, TLR ligation, such as via LPS, can induce the transcription of the inactive precursor of the pro-inflammatory cytokines, such as IL-1β, which is not constitutively present in cells (Franchi et al., 2009). These transcriptional changes prime the cell for subsequent inflammation, induced by a second family of PRRs.

This second family of PRRs is the NOD-like receptor (NLR) family. NLRs contain a nucleotide oligomerization and binding domain (NOD) (Lupfer and Kanneganti, 2013). There are at least 20 identified NLRs in humans (Correa et al., 2012). In addition to a nucleotide binding domain, such as a NACHT domain, NLRs typically contain a leucine rich repeat region (LRR) (Correa et al., 2012). These two core domains are supplemented by a wide variety of other domains that allow for protein-protein interactions (Martinon et al., 2009). By analogy to TLRs, the LRR was initially postulated as the NLR agonist binding region (Martinon et al., 2009). However, in most cases the binding partner remains unknown. NLR activation may be regulated by the NACHT domain, which is necessary for oligomerization of NLRs with other proteins during the inflammatory response (Martinon et al., 2009).

NLRs regulate many functions within the cell, including antigen recognition via MHC expression, damage and danger signal recognition, and overall regulation of inflammation through both positive and negative mechanisms (Lupfer and Kanneganti, 2013). For example, the NLRs NLRP3 and NLRC4 promote a robust pro-inflammatory response. However, the NLRs NLRC3, NLRP6, NLRP12, and NLRX1 are all known to play inhibitory roles in inflammation (Lupfer and Kanneganti, 2013). Other NLRs, including NLRP2 and NLRP7, regulate embryonic

development. For example, polymorphisms in NLRP7 are associated with miscarriage in humans (Huang et al., 2013). Thus, determining the mechanism through which NLRs promote or inhibit inflammation is crucial to understanding inflammation in both normal and pathological settings.

One way in which some NLRs promote inflammation is through the formation of multiprotein complexes called inflammasomes (Figure 1-1). These inflammasomes regulate the activation of pro-inflammatory caspases, such as caspase-1. Since each NLR contains a different combination of domains, each can interface with other proteins and caspase-1 in different ways. Generally, NLRs can either interact directly with caspase-1 via mutual caspase-recruitment domains (CARDs) or they may require a specific adaptor protein termed apoptosis-associated speck-like protein containing a CARD (ASC) (Martinon et al., 2009). ASC is a small protein consisting of a Pyrin domain and a CARD, enabling it to bridge the Pyrin domains of certain NLRs with caspase-1 (Martinon et al., 2009). Once this complex is formed, caspase-1 is activated. Caspase-1 is converted from its inactive pro-caspase form upon oligomerization in the inflammasome complex via ASC through auto-cleavage. This releases a p10 and p20 subunit of caspase-1, which can be detected as one measure of caspase-1 activation. Active caspase-1 can cleave pro-inflammatory cytokines such as pro-IL- $\beta$  and pro-IL-18 to their mature, active forms, which are then secreted from the cell. Caspase-1 also induces inflammatory programmed cell death, termed pyroptosis. Initiation of this cascade is dependent on each particular NLR in question.



**Figure 1-1: Examples of multiple inflammasomes**

NLRP1, NLRC4, NLRP3, and AIM2 inflammasomes are four common types of inflammasomes that can be activated in response to various stimuli. Structures are shown above. Activation of each inflammasome results in caspase-1 activation (Lamkanfi, 2011). Reprinted with permission.

### 1.2.1 NLRP1

NOD-like receptor family pyrin domain containing (NLRP) 1, also known as NALP1 or DEFCAP (Martinon et al., 2002; Moayeri et al., 2012), is one of the earliest identified NLRs and is known to form an inflammasome complex. NLRP1 consists of a nucleotide binding domain (NBD), function to find domain (FIIND), LRR, and a CARD. In humans, NLRP1 also contains a pyrin domain (Wen et al., 2013) and there are three known orthologs of NLRP1 in mice

(Schroder and Tschopp, 2010). Although NLRP1 can directly associate with caspase-1 via CARDs, ASC has been shown to enhance inflammasome function (Jin et al., 2013). This suggests that ASC can play a role in stabilization of an inflammasome in addition to acting directly as an adaptor molecule. NLRP1 can be activated by muramyl dipeptide and by *Bacillus anthracis* lethal toxin in mice (Faustin et al., 2007; Wen et al., 2013). The NLRP1 inflammasome shows one typical hallmark of inflammasome activation: caspase-1 dependent IL-1 $\beta$  processing and secretion. Interestingly, Nlrp1b deficient mice demonstrate an increase in pyroptosis, an inflammatory form of cell death, in a caspase-1 dependent manner (Wen et al., 2013). Like many inflammasomes, NLRP1 requires potassium efflux for inflammasome activation to occur (Fink et al., 2008).

### **1.2.2 NLRP12**

NLRP12, also known as Monarch-1, is one example of a non-inflammasome forming NLR (Pinheiro et al., 2011) that can interact with IRAK1 and TRAF3 (Lupfer and Kanneganti, 2013). It is found within myeloid cells, but the ligand is unknown (Latz et al., 2013). It is known that NLRP12 acts as a regulator of inflammation. First, NLRP12 can inhibit the NF- $\kappa$ B pathway (Lupfer and Kanneganti, 2013) and be a negative regulator of colon inflammation (Allen et al., 2012). It is also a positive regulator of dendritic cell migration (Latz et al., 2013). Finally, during *Yersinia pestis* infection, NLRP12 acts as a regulator of IL-1 $\beta$  and IL-18 release (Vladimer et al., 2012).

### 1.2.3 NLRC4

NOD-like receptor family, CARD containing (NLRC) 4, also known as Ipaf, forms an inflammasome that can sense bacterial products. NLRC4 can be activated by bacterial type III and IV secretion systems as well as the rod, needle, and flagellin from bacteria (Wen et al., 2013). NLRC4 activation in response to flagellin is distinct from the recognition of flagellin by TLR5 (Lamkanfi and Dixit, 2009). Prior to NLRC4 activation, NLR-family apoptosis inhibitory proteins (NAIPs) may initially interact with the components of these secretion systems and then activate NLRC4. For example, NAIP5 and 6 recognize flagellin, NAIP2 recognizes type III secretion system rod, and NAIP1 recognizes the type III secretion system needle (Kofoed and Vance, 2011; Wen et al., 2013; Yang et al., 2013; Zhao et al., 2011).

Consistent with the ability to recognize flagellin and bacterial secretion systems, the NLRC4 inflammasome is activated in response to *Salmonella typhimurium* and *Pseudomonas aeruginosa*, among other bacteria (Franchi et al., 2012; Schroder and Tschopp, 2010). It is thought that NLRC4 can discriminate between commensal and pathogenic bacteria in the intestine (Franchi et al., 2012). There is some controversy over whether NLRC4 requires phosphorylation for activation. Although active NLRC4 is phosphorylated (Wen et al., 2013), inactive NLRC4 can also be phosphorylated (Hu et al., 2013). As with other inflammasomes, NLRC4 inflammasome activation leads to IL-1 $\beta$  secretion and cell death. NLRC4 contains a CARD, suggesting that it does not require ASC for pyroptotic cell death, which has been demonstrated experimentally (Suzuki et al., 2007). Caspase-1 is required for pyroptotic cell death in response to NLRC4 activation (Vladimer et al., 2013).

#### 1.2.4 AIM2

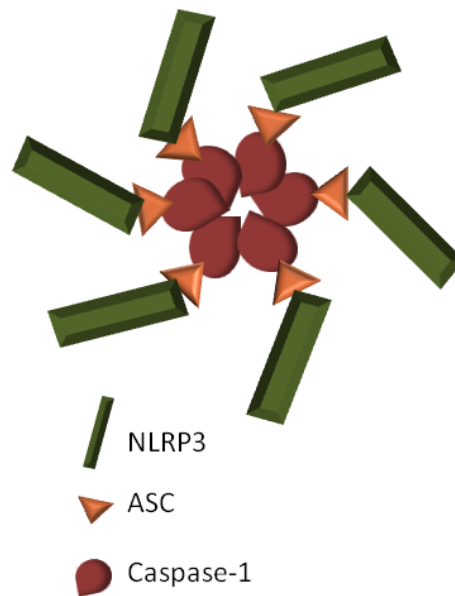
Although absent in melanoma 2 (AIM2) is not an NLR family member, it can also form an inflammasome. Instead of a nucleotide binding domain, AIM2 contains a Pyrin domain coupled to a HIN200 domain, placing it in the PYHIN family instead of NLR family. Through the Pyrin domain, AIM2 can interface with ASC and lead to the recruitment of caspase-1 with the expected IL-1 $\beta$  and IL-18 processing and secretion (Rathinam et al., 2010). The HIN200 domain enables AIM2 to bind to cytosolic dsDNA from bacterial, viral, or even host sources (Hornung et al., 2009; Muruve et al., 2008). Consequently, it is activated in response to pathogens that dwell in the cytosol, including murine cytomegalovirus, *Francisella tularensis*, and *Listeria monocytogenes* (Kim et al., 2010; Rathinam et al., 2010). The AIM2 inflammasome is crucial for host defense against bacterial infection. In AIM2<sup>-/-</sup> mice, the lack of a functional AIM2 inflammasome results in greater susceptibility to bacteria, such as *F. tularensis* (Fernandes-Alnemri et al., 2010). AIM2 is also a possible mediator of autoimmune diseases. AIM2 can respond to self DNA by promoting inflammation. Keratinocytes from psoriatic lesions were shown to have active AIM2 in response to host DNA, demonstrating a role for AIM2 in this autoinflammatory disease (Dombrowski et al., 2011).

#### 1.2.5 NLRP3

NLRP3, formerly known as NALP3 or cryopyrin, is one of the best characterized NLRs, and forms an inflammasome. NLRP3 is present in macrophages, dendritic cells, neutrophils, T cells, B cells, some epithelial cells, and pancreatic islet cells in both humans and murine models (Peeters et al., 2013; Zhou et al., 2010). NLRP3 consists of a NACHT domain, LRR, and a pyrin



domain (Martinon et al., 2009). Since NLRP3 lacks a CARD, it requires ASC for caspase-1 activation (Figure 1-2). It has been proposed that the binding of stimuli to the LRR region is responsible for NLRP3 inflammasome activation, however this is unproven. Given the structurally diverse array of stimuli (Table 1-1), it seems unlikely that a single ligand binding site could exist.



**Figure 1-2: NLRP3 inflammasome structure**

The NLRP3 inflammasome consists of NLRP3, ASC, and caspase-1. ASC acts as the adaptor molecule, connecting NLRP3 to caspase-1. Oligomerization of caspase-1 leads to caspase-1 activation and cleavage.

<b>Activator</b>	<b>Proposed signalling pathway</b>
<b>Microorganisms (PAMPs)</b>	
Sendai virus	ND
Influenza virus	ND
Adenovirus	ROS
<i>Candida albicans</i>	ROS
<i>Saccharomyces cerevisiae</i>	ROS
<i>Staphylococcus aureus</i>	ROS
<i>Listeria monocytogenes</i>	ND
Bacterial pore-forming toxins	ROS
<b>Endogenous danger signals (DAMPs)</b>	
Extracellular ATP	ROS and channel formation
Hyaluronan	ND
Glucose	ROS
MSU	ROS
Amyloid- $\beta$	Lysosome rupture
<b>Environmental irritants</b>	
Skin irritants	ROS
Imidazoquinoline compounds	ROS
Silica	ROS and lysosome rupture
Asbestos	ROS
Alum	ROS and lysosome rupture

**Table 1-1. Activators of the NLRP3 inflammasome**

Examples of common specific activators of the NLRP3 inflammasome with a wide range of sources and structures.

The second column proposes a common mediator of NLRP3 inflammasome activation, such as ROS, for each specific stimulus. modified from (Tschopp and Schroder, 2010) Reprinted with permission.

### 1.3 ACTIVATORS OF NLRP3

NLRP3 is activated by an incredibly diverse array of stimuli, including bacterial RNA, viral dsRNA, extracellular ATP, muramyl dipeptide, maitotoxin, amyloid beta, alum, silica crystals, calcium crystals, nigericin, potassium efflux, and pore-forming toxins (Blott and Griffiths, 2002; Boya and Kroemer, 2008; Chwieralski et al., 2006; Halle et al., 2008; Hentze et al., 2003; Petrilli et al., 2007; Zhao et al., 2005). It is unclear if NLRP3 senses these agonists directly or senses a common signal of cellular stress (Blott and Griffiths, 2002). These agonists can be broadly grouped by their function, as discussed below.

#### 1.3.1 Ionophores

One general category of NLRP3 inflammasome agonists are substances that lead to ion flux. The prototypical NLRP3 inflammasome agonist in this category is nigericin. Nigericin is a potassium ionophore produced by *Streptomyces hygroscopicus*. It triggers NLRP3 inflammasome but not other inflammasomes, and induces the processing and secretion of mature IL-1 $\beta$ . The exact mechanism of how nigericin activates NLRP3 is unclear, although it is likely to depend on the potassium ion efflux that it induces. This ion flux, and subsequent inflammasome activation, can be blocked through the addition of potassium chloride to cells. Early reports have suggested that potassium efflux is a common requirement for NLRP3 inflammasome assembly and its blockade could serve as an upstream inhibitor of all NLRP3 inflammasome dependent effects (Petrilli et al., 2007). Although the exact mechanism remains unclear, the requirement for potassium efflux does appear to be one common feature amongst the diverse repertoire of NLRP3 inflammasome activators (Kanneganti and Lamkanfi, 2013; Munoz-Planillo et al., 2013; Petrilli et al., 2007).

Calcium ionophores, such as calcimycin, can also activate the NLRP3 inflammasome. It has been proposed that the influx of calcium into cells may be required for NLRP3 inflammasome activation, possibly by triggering mitochondrial damage or reactive oxygen species (ROS) production (Murakami et al., 2012). However, the precise mechanism behind calcium as an NLRP3 agonist is poorly understood. In some studies, NLRP3 dependent ROS production preceded initial calcium flux (Zhong et al., 2013). Depleting cells of extracellular calcium with calcium free media or blocking calcium transport within the cells using pharmacological inhibitors has only had variable success in blocking NLRP3 inflammasome activation. The presence of intracellular calcium stores such as the endoplasmic reticulum (ER), mitochondria, and lysosomes further complicates interpretation of results, as it is possible that inflammasome activation may require release of calcium from only one organelle. Another factor complicating calcium-dependent NLRP3 activation is membrane repair. Most NLRP3 agonists directly damage the plasma membrane, and calcium-dependent cellular membrane repair (McNeil and Kirchhausen, 2005) would be necessary for the cell to survive long enough for inflammasome activation to occur. Moreover, it is difficult to exclude the importance of potassium efflux over the requirement for calcium influx during NLRP3 inflammasome activation, as the two frequently occur together. Thus, the role of calcium in NLRP3 activation remains unclear.

### **1.3.2 Pore-forming toxins**

In addition to ionophores that directly lead to ion flux, many stimuli of the NLRP3 inflammasome directly cause membrane damage which would also allow for potassium efflux. One large category of NLRP3 inflammasome agonists that allow potassium efflux are pore-

forming toxins (Gurcel et al., 2006). Several pore-forming toxins have been characterized as being activators of the NLRP3 inflammasome, including streptolysin O (SLO) from *Streptococcus pyogenes*, tetanolysin O (TLO) from *Clostridium tetani*, hemolysins produced by *Staphylococcus aureus*, Pneumolysin O (PLO) from *Streptococcus pneumoniae*, and ALO from *Bacillus anthracis* (Chu et al., 2009; Craven et al., 2009; Harder et al., 2009; McNeela et al., 2010; Shannon et al., 2003). Many bacterial pore-forming toxins, including SLO and TLO, bind to cell membrane cholesterol, and are termed cholesterol-dependent cytolysins (CDCs). CDCs are produced as soluble monomers containing four protein domains that can oligomerize on eukaryotic membranes to form pores up to 50 nm in diameter (Gonzalez et al., 2008). CDCs bind to the cholesterol on cell membranes through a conserved, tryptophan rich undecapeptide region present in domain four of the molecule (Tweten, 2005). Up to 50 monomers can form a pore and oligomerization, which is dependent on toxin concentration, is the rate determining step (Gilbert, 2002; Gonzalez et al., 2008). Initially, a pre-pore complex is formed on the membrane, which, once it reaches a large enough size, transforms into a fully formed pore (Rossjohn et al., 1997). This transition occurs when the two alpha helices present in domain 3 become two extended  $\beta$  hairpins (Tweten, 2005). If the plasma membrane is depleted of cholesterol, the pre-pore is prevented from transitioning to the pore complex (Tweten, 2005).

In addition to cell lysis, CDCs may have other effects on the cell. The effect of pore formation on the cell varies depending on the toxin dose, the duration of the exposure, and the cell type. The dose of toxin that cells are exposed to during infection has not been determined, but is thought to vary based on the distance between the target cell and the site of infection (Gonzalez et al., 2008). CDCs can allow the entry of bacterial virulence factors into the host cell in a process has been termed cytolysin-mediated translocation (Bricker et al., 2002; Shannon et al., 2003). Furthermore, CDCs can be used as a

research tool to transfer proteins into living cells (Gonzalez et al., 2008). However, for NLRP3 inflammasome activation, SLO mutants with reduced lytic capacity increased secretion of IL-1 $\beta$ , suggesting that cytokine release was not merely a reflection of cell disruption (Keyel et al., 2013).

The CDC toxin TLO is produced during infection by *Clostridium tetani*, although it may be less important to virulence than SLO during an infection with *Streptococcus pyogenes* (Keyel et al., 2013; Limbago et al., 2000). *C. tetani* is a spore-forming, gram positive bacterium that is typically found in the soil and animal feces (Hsu and Groleau, 2001). *C. tetani* is a bacillus and, although an obligate anaerobe, it is somewhat tolerant of an aerobic environment, possibly due to the presence of heme oxygenase (Bruggemann et al., 2004; Finegold, 1995). *C. tetani* typically enters the host through an open wound and produces two toxins: tetanospasmin and TLO (Hsu and Groleau, 2001). Tetanospasmin enters the central nervous system where it disables the inhibitory neurotransmitter GABA, which results in muscle spasms (Hsu and Groleau, 2001). The resulting disease is known as tetanus and remains a problem worldwide. Tetanus can be treated through the administration of human tetanus immunoglobulin which neutralizes tetanospasmin that has not yet entered the central nervous system. Antibiotics, such as metronidazole, are given to kill the bacteria and GABA agonists are administered to prevent muscle spasms (Hsu and Groleau, 2001). The damage inflicted by the other toxin, TLO, has remained unexplored until recently, and therefore, no treatment targets this toxin. This fact stresses the importance of determining how this toxin affects cells, specifically macrophages, which are needed to clear the *C. tetani* infection. If the mechanism of TLO action were better understood, drugs could be developed to neutralize its effects. Like other pore-forming toxins, TLO induces NLRP3 inflammasome activation, though the precise mechanism for pore-forming toxins remains unknown (Chu et al., 2009).

### 1.3.3 Particulate Structures

A third group of NLRP3 agonists are crystals or other particulate structures. These include substances like silica, alum, monosodium urate (MSU), asbestos, cholesterol crystals, prion fibrils, and amyloid beta (Dostert et al., 2008; Halle et al., 2008; Hornung et al., 2008; Martinon et al., 2006; Rajamaki et al., 2010). A common feature among these stimuli may be the involvement of lysosomal disruption. Since lysosomes degrade many cellular components, they contain many acidic proteases, including the cysteine proteases termed cathepsins. Although most cathepsins lose proteolytic activity at a neutral pH, cathepsin B retains partial activity at a neutral pH. Therefore, although it is optimally active within the acidic environment of the lysosome, cathepsin B retains activity in the neutral cytosol if leaked out during lysosomal damage. Silica and aluminum crystals, for example, appear to activate the NLRP3 inflammasome through a lysosomal damage dependent pathway. NLRP3 dependent IL-1 $\beta$  secretion was blocked by an inhibitor of the lysosomal protease cathepsin B, lysosomal acidity, and by phagocytosis inhibitors (Halle et al., 2008; Hornung et al., 2008; Sharp et al., 2009). However, the ability of cathepsin B inhibitors to block NLRP3 inflammasome activation may depend on the specific stimulus, and may not be universal. In addition, the cathepsin B inhibitor, CA-074 ME has been shown to have off-target effects, and cathepsin B<sup>-/-</sup> mice have no defects in inflammasome activation in some studies (Dostert et al., 2009; Newman et al., 2009). Therefore, a role for cathepsin B in NLRP3 inflammasome activation is still uncertain.

Other forms of lysosomal disruption, such as through treatment with L-leucyl-L-leucine methyl ester (Leu-Leu-OMe), or rupture by exposing cells to a hypotonic solution, also activate the NLRP3 inflammasome (Hornung et al., 2008). Therefore, the release of lysosomal DAMPs could be the general NLRP3 inflammasome agonist. Crystals would facilitate DAMP release

through mechanical disruption of the lysosomes via “frustrated phagocytosis” (Martinon et al., 2009). ROS production and subsequent IL-1 $\beta$  secretion both occurred following treatment of cells with crystals (Dostert et al., 2008; Martinon, 2010; Sautin et al., 2007). In this model, particle size and surface features would be predicted to later inflammasome activation kinetics. However, there has been some debate whether particle size and surface features play a role in NLRP3 inflammasome activation (Adachi et al., 2013; Morishige et al., 2010).

#### **1.3.4 Reactive Oxygen Species**

A fourth general category of cellular stressor that is associated with NLRP3 inflammasome activation is ROS production. ROS generation has been detected during NLRP3 inflammasome stimulation from ATP, nigericin, crystals, and other NLRP3 agonists (Cruz et al., 2007; Dostert et al., 2008; Zhou et al., 2010). ROS scavengers and inhibitors block NLRP3 inflammasome activation (Pedra et al., 2009). Both lysosomes and mitochondria are two major sources of ROS that have been reported. Some groups have suggested that lysosomal ROS is responsible for activating the inflammasome, but this remains controversial. It is more likely that ROS generation occurs as a result of lysosomal damage or disruption rather than directly from the lysosome itself. For example, lysosomes are a source of redox-active free iron, which can be involved with Fenton-like reactions and ROS generation (Hamacher-Brady et al.). Although NADPH oxidases have been suggested as potential sources of ROS (Dostert et al., 2008), macrophages deficient in the NOX1, NOX2, or NOX4 subunits of NADPH oxidase had normal inflammasome activation (Zhou et al., 2011). Furthermore, there have been some reports, especially in chronic granulomatous disease, where NADPH oxidase generated ROS suppresses inflammation (Latz, 2010; Meissner et al., 2010; Segal et al., 2010).



#### 1.3.4.1 Mitochondria as a source of ROS

Another possible source of cellular ROS are mitochondria. Mitochondria are necessary for production of ATP for cellular energy. However, they are also involved with lipid metabolism, amino acid turnover, cell death, and inflammation. The mitochondria produce the majority of cellular ATP via oxidative phosphorylation through the reduction of oxygen via complexes I through V (Sorbara and Girardin, 2011). Complex I and IV are involved with proton pumping (Scheffler, 2001). Complex V is the ATP synthase. As a result, mitochondria are a major source of ROS, including  $O_2^-$ ,  $H_2O_2$ , and hydroxyl radical (Sorbara and Girardin, 2011). The mitochondria consist of an outer membrane and an inner membrane, which folds to form the cristae, where most complexes of oxidative phosphorylation are located (Youle and van der Bliek, 2012). Mitochondria are very dynamic organelles, constantly moving and “dividing.” Mitochondrial movement has been observed in both retrograde and anterograde directions (Scheffler, 2001). This motility depends not only on actin and microtubules, but also other organelles. Mitochondria can undergo both fission and fusion. Fission is used to create mitochondrial fragments and is associated with mitophagy (Hernandez-Aguilera et al., 2013). Fusion on the other hand, interconnects mitochondria and maximizes ATP synthesis (Hernandez-Aguilera et al., 2013). Damaged mitochondria that are unable to produce ATP lose their fusion and fission machinery, thus preventing damage to other healthy mitochondria (Youle and van der Bliek, 2012). Typically, healthy mitochondria remain in a polarized state due to the transport of electrons across their membrane. Ions cross the inner mitochondrial membrane via antiport mechanisms. Potassium ions can enter the mitochondrion via  $K^+/H^+$  antiporter, ATP regulated  $K^+$  channel, or based on the polarization state of the mitochondrion (Scheffler, 2001). Calcium, sodium, and iron ions also are able to cross the mitochondrial membrane.

Mitochondria can alter their permeability through membrane permeability transition (MPT), which requires the opening of the permeability transition pore (PTP). The PTP consists of a voltage dependent anion channel (VDAC) in the outer mitochondrial membrane, adenine nucleotide transporter, and cyclophilin D (Tsujimoto and Shimizu, 2007). Pore opening can be triggered by various stimuli, such as calcium ions, mitochondrial depolarization, and potassium efflux (Scorrano et al., 1997; Tsujimoto and Shimizu, 2007). PTP opening can be inhibited by cyclosporine A, which binds cyclophilin D. The anti-apoptotic protein Bcl-2 also inhibits the pore opening (Tsujimoto and Shimizu, 2007). Mitochondrial swelling, depolarization, and breakdown of the mitochondrial membrane result from the opening of the PTP (Tsujimoto and Shimizu, 2007).

When mitochondria depolarize or experience metabolic dysfunction, ROS are produced (Zhou et al., 2011). This ROS production can occur as a result of disruption of complex I or III in the electron transport chain, as seen with the complex I inhibitor rotenone or complex III inhibitor antimycin A (Zhou et al., 2011). Bcl-2 dependent inhibition of VDAC 1 and 2 also increases mitochondrial ROS production (Zhou et al., 2011). Inhibition of VDAC reduced both ROS production and NLRP3 inflammasome activation (Zhou et al., 2011). Similarly, inhibition of PTP opening blocks IL-1 $\beta$  release in response to ATP stimulation of the NLRP3 inflammasome (Kepp et al., 2011). Increased levels of mitochondrial generated ROS are positively associated with higher levels of NLRP3 inflammasome dependent IL-1 $\beta$  secretion in human macrophages (Naik and Dixit, 2011; Zhou et al., 2011). The use of dyes and inhibitors that are specific for mitochondrial generated ROS have confirmed that mitochondria are the source for ROS dependent NLRP3 inflammasome activation (Nakahira et al., 2011). For example, treatment with the mitochondrial ROS scavenger MitoTEMPO inhibits NLRP3 dependent IL-1 $\beta$  processing and secretion in response to multiple NLRP3 agonists, including nigericin

and ATP (Nakahira et al., 2011). These results suggest that mitochondrial ROS are important for NLRP3 inflammasome activation.

One possible mechanism through which ROS generation can activate the NLRP3 inflammasome involves thioredoxin interacting protein (TXNIP). Under conditions of oxidative stress, TXNIP dissociates from its binding partner, thioredoxin (TRX) when TRX is oxidized (Zhou et al., 2010). TRX is then free to act as an ROS scavenger (Schroder et al., 2010). TXNIP binds to NLRP3 during H<sub>2</sub>O<sub>2</sub> or uric acid induced ROS generation in human macrophages, leading to caspase-1 cleavage and IL-1 $\beta$  secretion (Zhou et al., 2010). NLRP3 inflammasome, but not NLRC4 or AIM2 inflammasome, dependent cytokine secretion was enhanced with over expression of TXNIP and reduced in TXNIP<sup>-/-</sup> cells (Zhou et al., 2010). Therefore, this is one mechanism through which ROS generation in cells, possibly of mitochondrial and/or lysosomal origin, could either initiate or perpetuate NLRP3 inflammasome activation. Furthermore, TXNIP mRNA levels are increased during the unfolded protein response in ER stress, leading to inflammation and cell death (Anthony and Wek, 2012; Lerner et al., 2012; Osowski et al., 2012). This has been shown to be relevant in a type 2 diabetes model, where TXNIP is upregulated (Zhou et al., 2010).

ROS have a short half-life in cells due to inactivation by ROS scavengers. The short half-life of ROS suggests that there is a rapid mechanism for mitochondrial ROS to activate the NLRP3 inflammasome. Several recent reports have suggested that the NLRP3 inflammasome is in close proximity to the mitochondria, where it can be rapidly activated by mitochondrial ROS. Although NLRP3 is not known to be directed to the mitochondria like NLRX 1 (Moore et al., 2008; Tattoli et al., 2008), NLRP3 co-localizes with the mitochondria via both microscopy and fractionation. In unstimulated cells, NLRP3 associates with the ER, while ASC is cytosolic. Upon stimulation with either MSU, nigericin, or alum, NLRP3 and ASC associated with a mitochondrial/ER interface, with

ASC more predominant in the mitochondrial fraction and NLRP3 in the ER fraction (Zhou et al., 2011). This region potentially corresponds to the mitochondrial associated membranes (MAMs), which are responsible for calcium exchange from the ER to the mitochondria (Hayashi et al., 2009).

Further evidence that the NLRP3 inflammasome forms at the mitochondria comes from a second study where NLRP3 was found to be associated with both ASC and the mitochondria-associated adaptor molecule mitochondrial anti-viral signaling protein (MAVs) (Subramanian et al., 2013). This was confirmed more recently by Park et al, who showed that the C-terminal domain of MAVs was essential for NLRP3 inflammasome formation during viral infection (Park et al., 2013). Finally, Misawa et al. (2013), found that ASC-bearing mitochondria are transported to NLRP3-containing ER via microtubules (Misawa et al., 2013). This dynein dependent transport requires decreased levels of  $\text{NAD}^+$  in cells, possibly due to mitochondrial damage, leading to an accumulation of acetylated  $\alpha$  tubulin (Misawa et al., 2013). This study also offered a mechanism through which a microtubule polymerization inhibitor, colchicine, blocks NLRP3 inflammasome mediated  $\text{IL-1}\beta$  secretion (Martinon et al., 2006). Moreover, TXNIP associates with mitochondria and NLRP3 (Zhou et al., 2011), lending further support for TXNIP-dependent, mitochondrial ROS mediated activation of the NLRP3 inflammasome. In pancreatic islet cells, TXNIP translocates from the nucleus to the mitochondria upon oxidation, and associates with mitochondrial Trx2 (Saxena et al., 2010). Trx2 then dissociates from ASK1, which leads to the mitochondrial death pathway (Saxena et al., 2010). These findings emphasize the important role for the mitochondria in not only pro-inflammatory cytokine production, but also in cell death.

### 1.3.5 Other NLRP3 agonists from the mitochondria

In addition to mitochondrial ROS, there are other potential mitochondrially-derived NLRP3 agonists. One such agonist is cardiolipin, which is an inner mitochondrial membrane phospholipid. This phospholipid can bind to the NLRP3 inflammasome. This interaction is required for the antibiotic linezolid to induce NLRP3 inflammasome activation. Interestingly, this interaction does not require the generation of ROS (Iyer et al., 2013).

Mitochondrial DNA (mtDNA) is another potential activator of the NLRP3 inflammasome. Since mitochondria are of  $\alpha$ -purple bacterial origin, mtDNA contains hallmarks of bacterial DNA, including CpG repeats (Taanman, 1999; Zhang et al., 2010). As a result, mtDNA is a DAMP recognized by immune cells (Zhang et al., 2010). Mitochondrial DNA can bind to the NLRP3 inflammasome following mitochondrial damage and apoptotic cell death (Shimada et al., 2012). Oxidation of mtDNA further enhances NLRP3 dependent IL-1 $\beta$  secretion over that induced by nonoxidized mtDNA. This may be a result of oxidized DNA in general being more resistant to degradation and better able to act as a DAMP (Gehrke et al., 2013). However, both nonoxidized and oxidized mtDNA can bind to NLRP3, indicating a potential regulatory mechanism that requires both mtDNA and ROS to be present for NLRP3 inflammasome activation to occur (Martinon, 2012; Shimada et al., 2012). Mitochondrial ROS may be responsible for the oxidation of mtDNA in both ATP and nigericin treated cells (Shimada et al., 2012). Similarly, mitochondrial ROS generation is required for the NLRP3 dependent release of mtDNA into the cytosol of ATP treated macrophages (Nakahira et al., 2011). However, NLRP3 is also required for PTP opening, which is required for the release of mtDNA. Thus, NLRP3 inflammasome activation could be triggered by mitochondrial dysfunction, such as ROS production, and then enters a positive feedback loop through increased mitochondrial damage which further enhances mtDNA leakage (Kepp et al., 2011).

## 1.4 MITOPHAGY

Due to the widespread deleterious effects that damaged mitochondria can exert on both the NLRP3 inflammasome, and the cell in general, the removal of damaged mitochondria by the cell is imperative for continued cell survival. Autophagy is the turnover of damaged organelles and cellular components in a controlled process of “self eating.” It is associated with the formation of LC3 punctae in the cell and double membrane structures. Mitophagy refers to the autophagy of mitochondria (Nakahira et al., 2011). The inhibition of autophagy in general has been associated with enhanced NLRP3 inflammasome activation. Presumably this occurs through the accumulation of damaged or dysfunctional ROS producing mitochondria in the cell that are also leaking mtDNA. Deletion of autophagic proteins prevents the removal of these damaged mitochondria. Since the products of damaged mitochondria lead to NLRP3 inflammasome activation, this explains why cells lacking the autophagy protein Atg16L1, or those treated with inhibitors of autophagy, have increased levels of IL-1 $\beta$  (Harris et al., 2011; Saitoh et al., 2008). Higher levels of IL-1 $\beta$  secretion are also associated with deletion of other autophagy proteins LC3B and Beclin 1. Furthermore, the inhibition of mitophagy through the deletion of LC3B and Beclin 1 increased the amount of mitochondrial dysfunction and mitochondrial ROS generation in cells, thus enhancing inflammasome activation and mtDNA release (Nakahira et al., 2011). Thus, there is a tight balance between autophagy, NLRP3 inflammasome activation, inflammation and cell death.

## 1.5 PYROPTOSIS

Inflammasome activation leads to cell death through a process termed pyroptosis. Pyroptosis is an inflammatory form of programmed cell death, initially defined as requiring the activity of caspase-1 (Bergsbaken et al., 2009). Unlike apoptotic cell death, pyroptosis involves the release of inflammatory cellular contents, cell swelling and rupture, and the formation of 1.1-2.4 nm pores in the plasma membrane (Bergsbaken et al., 2009). Pyroptosis has been observed in response to activation of all known inflammasome complexes (Bortoluci and Medzhitov, 2010). It is commonly seen in response to bacterial pathogens, including *Salmonella*, *Francisella novicida*, and *Legionella* (Bergsbaken et al., 2009). However, the benefit for pyroptotic cell death in bacterial clearance is not well understood. A host benefit for pyroptotic cell death is unknown in response to NLRP3 or NLRP1b inflammasome activation. Only activation of the NLRC4 inflammasome has been associated with enhanced bacterial clearance (Bortoluci and Medzhitov, 2010). Whether this is due directly to cell death or rather alternative mechanisms, such as caspase-7 activation or iNOS induction, is unknown (Akhter et al., 2012). Furthermore, caspase-1 activity was shown to enhance bacterial clearance in response to *F. novicida* infection, even in mice lacking IL-1 $\beta$  and IL-18 (Henry et al., 2007). It has been proposed that caspase-1 induced pyroptotic cell death during *Salmonella* infection assists in bacterial clearance through the release of bacteria from the host macrophages so that neutrophils can then kill the extracellular bacteria (Miao et al., 2010). Caspase-1 dependent pyroptotic cell death is distinct from inflammasome mediated cytokine release. Cell lysis is not required for IL-1 $\beta$  release (Fink and Cookson, 2006) and neither IL-1 $\beta$  nor IL-18 is required for pyroptosis (Le Feuvre et al., 2002; Monack et al., 2001). Furthermore, the loss of mitochondrial integrity and release of cytochrome c, which can

induce mitochondrial mediated cell death, are not required for pyroptotic cell death (Bergsbaken et al., 2009).

Although pyroptosis was initially described as a caspase-1 dependent process, the caspase-1<sup>-/-</sup> mice used to dissect this mechanism also lacked caspase-11. Due to this discovery, more studies have been conducted to delineate the requirement for each caspase in systems previously only thought to require caspase-1 (Viganò and Mortellaro, 2013). Caspase-11 mediates a noncanonical inflammasome pathway that induces cell death. Stimuli of this pathway include *Escherichia coli*, cholera toxin B, *Citrobacter rodentium*, and *Vibrio cholerae* (Viganò and Mortellaro, 2013). Pro-caspase-11 is induced by TLR ligands such as LPS, and pro-caspase-11 can activate itself through an autocatalytic event (Kang et al., 2000; Rathinam et al., 2012). In response to noncanonical stimuli, caspase-11 deficiency results in increased survival and lack of DAMP release from cells (Kayagaki et al., 2011). Caspase-11 has been proposed to play a role in bacterial clearance by facilitating phagosome fusion with the lysosome (Akhter et al., 2012). However, caspase-11 mediated cell death, in the absence of caspase-1, was found to enhance disease during *Salmonella* infection (Broz et al., 2012). Caspase-11 inflammasome function is distinct from NLRP3, since NLRP3 and ASC are not required for the activity of caspase-11. Conversely, caspase-11 is not required for NLRP3 or NLRC4 induced pyroptotic cell death (Viganò and Mortellaro, 2013).

## 1.6 NONCANONICAL ROLES OF ASC

In addition to noncanonical inflammasomes, there are also noncanonical, inflammasome independent roles for the adaptor protein ASC. Most notably, during *Legionella pneumophila*



infection of macrophages, there is evidence that ASC can act independently of NLRC4 to activate caspase-1 and caspase-3 (Abdelaziz et al., 2011). ASC can regulate Dock2, a nucleotide exchange factor that regulates actin polymerization (Ippagunta et al., 2011). This regulates the GTPase Rac, which affects dendritic cell phagocytosis and migration of T and B cells (Ippagunta et al., 2011). Furthermore, ASC regulates MAPK/ERK2 activity independently of caspase-1 (Hassan and Amer, 2011; Taxman et al., 2011). Finally, ASC is responsible for the adjuvant effect of MF59 in flu vaccines, independently of NLRP3 and caspase-1 (Ellebedy et al., 2011). The production of IgG antibodies requires ASC and bone marrow derived macrophages secrete lower levels of macrophage inflammatory protein (MIP1b and MIP2) in the absence of ASC (Ellebedy et al., 2011). These findings suggest a role for ASC in the creation of a pro-inflammatory environment independently of the inflammasome.

## **1.7 IL-1 BETA AND AUTOINFLAMMATORY DISEASE**

The primary mediators of the inflammation following inflammasome-dependent release are the IL-1 family members IL-1 $\beta$  and IL-18. IL-1 $\beta$  is the founding member of a protein family containing at least eleven known members (Dinarello, 2009). IL-1 family members can be divided into pro-inflammatory and anti-inflammatory groups. In addition to IL-1 $\beta$  and IL-18, pro-inflammatory members include IL-1 $\alpha$ , IL-1H<sub>2</sub>, and IL-33 (Dinarello, 2009). The IL-1 receptor antagonist (IL-1Ra) and IL-1Hy2 are antagonists of the IL-1 receptor and IL-18BP inhibits IL-18 (Dinarello, 2009; Petrusek et al., 2012).

Unprocessed, biologically inactive, IL-1 $\beta$  exists as a 33kDa precursor in the cytoplasm at low levels in unstimulated cells (Dubyak, 2012). Upon stimulation with PAMPs such as LPS,

cells upregulate mRNA for pro-IL-1 $\beta$  as quickly as 15 minutes after stimulation (Dinarello, 2009). This upregulation of pro-IL1 $\beta$  requires NF- $\kappa$ B activity (Franchi et al., 2009). The normally short half life of this mRNA can be extended if cells are stimulated by IL-1 in a positive feedback loop (Dinarello, 2009). Pro-IL-1 $\beta$  must be processed to its biologically active, 17kDa form (Dubyak, 2012). Although this processing can occur via caspase-1 in the context of inflammasome activation within the cell, there are other mechanisms for IL-1 $\beta$  processing (Dubyak, 2012). Neutrophil protease proteinase-3 (Coeshott et al., 1999), elastase, matrix metalloprotease 9, and granzyme A can also process IL-1 $\beta$  (Dinarello, 2009).

The mechanism through which mature IL-1 $\beta$  is secreted from the cell after processing remains unknown. IL-1 $\beta$  lacks a signal sequence that would target it to the Golgi for classical packaging and secretion, and is usually processed in the cytoplasm (Dubyak, 2012). IL-1 $\beta$  is secreted via a non-classical secretion pathway that also involves the secretion of caspase-1 and ASC (Dubyak, 2012; Rubartelli et al., 1990). This non-classical secretion mechanism is not unique to IL-1 $\beta$ , as the IL-1 family members IL-1 $\alpha$  and IL-18 also undergo non-classical secretion (Gross et al., 2012; Mariathasan et al., 2004).

There are three main hypotheses to explain how IL-1 $\beta$  is non-classically secreted from the cell. First, secretory lysosomes have been proposed as a means for transporting IL-1 $\beta$  out of monocytes in response to ATP stimulation (Dubyak, 2012). In support of this theory, lysosomal cathepsins, such as cathepsin B, are secreted with IL-1 $\beta$  by both mouse and human monocytes (Carta et al., 2006; Qu et al., 2007). Inhibition of microtubule polymerization, which disrupts lysosomal traffic along microtubules, also inhibits IL-1 $\beta$  secretion (Carta et al., 2006). Although there are other explanations for this result, it does not exclude the possibility that lysosomal traffic along microtubules is required for the secretion of IL-1 $\beta$ . A second hypothesis for

explaining non-classical IL-1 $\beta$  secretion is through the secretion of IL-1 $\beta$  containing microvesicles or exosomes (Dubyak, 2012). Microvesicles, which are vesicular structures characterized as being 100 nm to 1  $\mu$ m in diameter, have been proposed to carry not only IL-1 $\beta$ , but also IL-18 and DAMPs, from ATP treated cells (Gardella et al., 2002). Exosomes, which are 50-100 nm diameter, cup-shaped, vesicles released from multivesicular bodies, have also been suggested as a means for exporting IL-1 $\beta$ , caspase-1 and ASC out of the cell (Dubyak, 2012). Finally, autophagosomes or autophagolysosomes have been proposed as mediators of IL-1 $\beta$  release from cells (Dubyak, 2012). In support of this hypothesis, the NLRP3 inflammasome is ubiquitinated for degradation following its activation (Shi et al., 2012). Autophagosomes form at the site of IL-1 $\beta$  processing to degrade the ubiquitinated inflammasome, as well as any damaged mitochondria associated with the NLRP3 inflammasome complex (Nakahira et al., 2011). Although these autophagosomes normally fuse with lysosomes to form autophagolysosomes for the degradation of NLRP3 and damaged mitochondria, they could be redirected to the plasma membrane for IL-1 $\beta$  secretion instead (Dubyak, 2012). Also supporting this hypothesis is the association of IL-1 $\beta$  with the autophagosome protein LC3B (Takenouchi et al., 2009).

Regardless of the secretion mechanism, secreted IL-1 $\beta$ , as well as other IL-1 family members, have numerous effects on cells. IL-1 $\beta$  signals through the IL-1 receptor (IL-1R) and can lead to NF- $\kappa$ B and MAPK activation (Lamkanfi and Dixit, 2009). IL-1 $\beta$  induces the gene expression of cyclooxygenase 2 (COX-2) and iNOS, leading to PGE2 and NO production (Dinarello, 2009). IL-1 $\beta$  also increases the expression of adhesion molecules, allowing for the infiltration of inflammatory cells (Dinarello, 2009; Franchi et al., 2009). Additionally, IL-1 $\beta$  leads to fever, promotes T cell activation, Th2 polarization, and assists in antibody production in response to T dependent antigens (Dinarello, 2009; Lamkanfi and Dixit, 2009; Martinon et al.,

2009). Finally, IL-1 $\beta$  plays a role in increasing ovulation and decreasing embryonic development in mares and rabbits (Caillaud et al., 2005; Takehara et al., 1994).

Due to its many functions, it is not surprising that IL-1 $\beta$  plays a role in many inflammatory diseases. The main features of IL-1 $\beta$  related diseases are fever, hypersensitivity to pain, and inflammation (Martinon et al., 2009). In addition to cancer, diabetes, atherosclerosis, rheumatoid arthritis, and lupus, there are several diseases involving mutations in NLRP3. These mutations cause hyperactive inflammasome function which leads to high levels of IL-1 $\beta$  (Agostini et al., 2004; Martinon et al., 2009). These autoinflammatory diseases, frequently associated with recurrent fevers, include familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal onset multisystem inflammatory disease (Martinon et al., 2009). These diseases are commonly treated with IL-1 receptor antagonists (Martinon et al., 2009). However, inflammasome activation leads to many other pro-inflammatory processes. Therefore, blocking inflammasome function further upstream is a desirable therapeutic target. The ability to specifically target individual inflammasomes is important to maintain resistance to disease during therapy. However, in order to block inflammasome function, the mechanism of NLRP3 activation must first be understood.

## **1.8 PURPOSE OF THESIS WORK**

Although many activators of the NLRP3 inflammasome have been identified, the exact mechanism of activation remains unknown. There are numerous cellular events that are associated with inflammasome activation that have been characterized. However, the kinetics of these events, and which events initiate or are a result of inflammasome activation are not clear.

The work described in the next chapters has expanded the knowledge of NLRP3 inflammasome activation in relation to two subcellular organelles, mitochondria and lysosomes. This work resolves the conflict on the importance of lysosomes versus mitochondria during inflammasome activation, placing lysosomal membrane permeability (LMP) as a downstream event of NLRP3 inflammasome activation, and characterizing the ROS driven sequence of events that occur during NLRP3 inflammasome activation. The knowledge presented here can further the field of inflammasome research by providing details of the NLRP3 inflammasome activation pathway that are valuable for therapeutic manipulation.

First, my data have explored the role of both the lysosomes and the mitochondria during NLRP3 inflammasome activation. As previously discussed, numerous studies have favored a role for either the lysosome or the mitochondria during NLRP3 inflammasome activation. However, very few studies have suggested a role for both organelles in inflammasome activation. Here, I propose, and provide data supporting, crosstalk between mitochondria and lysosomes during nigericin induced NLRP3 inflammasome activation. This crosstalk is mainly mediated through ROS production. My data suggest that dysfunctional mitochondria produce mitochondrial ROS in an NLRP3 inflammasome independent manner which then activate the NLRP3 inflammasome and induce lysosomal damage. NLRP3 dependent lysosomal damage and further production of ROS disrupt the mitochondrial membrane in an NLRP3 inflammasome dependent manner, leading to cell death.

Second, I have placed LMP downstream of NLRP3 inflammasome activation. Although lysosomal damage during frustrated phagocytosis is postulated as a trigger of NLRP3, my data suggest LMP occurs after NLRP3 inflammasome activation in response to nigericin, ATP, and TLO. NLRP3<sup>-/-</sup> macrophages did not undergo LMP in response to NLRP3 inflammasome

stimuli, unlike WT cells. Furthermore, although IL-1 $\beta$  secretion was dependent on the lysosomal protease cathepsin B, other NLRP3 dependent events did not require cathepsin B activity.

Finally, my data have demonstrated the importance of ROS production, mainly from the mitochondria, as a key player in NLRP3 inflammasome activation. IL-1 $\beta$  processing and secretion were dependent on mitochondrial ROS production. Furthermore, LMP and mitochondrial membrane permeability were either reduced or delayed in the presence of an inhibitor of mitochondrial ROS. Taken together, my data extend previous work suggesting that mitochondrial ROS is required for NLRP3 inflammasome activation by explaining how mitochondrial ROS influence LMP. I further suggest that in addition to IL-1 $\beta$  secretion, lysosomal and mitochondrial damage are influenced by mitochondrial ROS production. Thus, I present mitochondrial ROS as a broader target than IL-1 $\beta$  production for therapeutic approaches to inflammatory diseases.

## **2.0 MITOCHONDRIAL ROS INDUCES NLRP3-DEPENDENT LYSOSOMAL DAMAGE AND INFLAMMASOME ACTIVATION**

### **2.1 AUTHORS AND THEIR CONTRIBUTIONS**

This study was published in the *Journal of Immunology* (published ahead of print October 2, 2013, doi: 10.4049/jimmunol.1301490; Copyright 2013. The American Association of Immunologists, Inc.). Michelle E. Heid (Department of Immunology, University of Pittsburgh) wrote the manuscript, designed experiments, conducted the majority of the experiments, and analyzed most of the data. Peter A. Keyel (Department of Immunology, University of Pittsburgh) assisted with live cell microscopy analysis. Christelle Kamga and Sruti Shiva (Department of Pharmacology and Chemical Biology, University of Pittsburgh) provided useful discussion of mitochondrial metabolism. Simon C. Watkins (Department of Cell Biology, University of Pittsburgh) assisted with live cell microscopy and provided technical advice and helpful discussion throughout the project. Russell D. Salter (Department of Immunology, University of Pittsburgh) was the Principle Investigator for the project, provided mentorship, advice, and assistance with writing throughout the entire project. I would also like to acknowledge Lisa Borghesi and Dr. Olivera Finn (Department of Immunology, University of Pittsburgh) for providing WT bone marrow and Tim Billiar (Department of Surgery, University of Pittsburgh) for providing NLRP3<sup>-/-</sup> bone marrow.

## 2.2 ABSTRACT

The NLRP3 inflammasome drives many inflammatory processes and mediates IL-1 family cytokine release. Inflammasome activators typically damage cells, and may release lysosomal and mitochondrial products into the cytosol. Macrophages triggered by the NLRP3 inflammasome activator nigericin show rapid depolarization of mitochondria and reduced cellular ATP. Release of mitochondrial ROS leads to subsequent lysosomal membrane permeabilization (LMP). NLRP3-deficient macrophages show comparable mitochondrial depolarization and ATP loss, but maintain lysosomal acidity, demonstrating that LMP is NLRP3 dependent. A subset of WT macrophages undergo subsequent mitochondrial membrane permeabilization (MMP) and die. Both LMP and MMP are inhibited by potassium, scavenging mitochondrial ROS, or NLRP3 deficiency, but are unaffected by cathepsin B or caspase-1 inhibitors. In contrast, IL-1 $\beta$  secretion is ablated by potassium, scavenging mitochondrial ROS, and both cathepsin B and caspase-1 inhibition. These results demonstrate interplay between lysosomes and mitochondria that sustain NLRP3 activation, and distinguish cell death from IL-1 $\beta$  release.

## 2.3 INTRODUCTION

Inflammasome activation plays an integral part of the innate immune response in host defense and in other inflammatory diseases. The NOD-like receptor family, pyrin domain-containing three (NLRP3) inflammasome is present in a variety of cells, including macrophages, dendritic cells, neutrophils, T cells, B cells, and some epithelial cells (Petrilli et al., 2007). The NLRP3



inflammasome is a protein scaffolding complex consisting of NLRP3, caspase-1, and the adaptor molecule ASC (Pycard) that induces secretion of IL-1 family cytokines, including interleukin-18 (IL-18) and interleukin-1 beta (IL-1 $\beta$ ) (Agostini et al., 2004; Dubyak, 2012). The processing of IL-1 $\beta$  from biologically inactive precursor to the active form requires cleavage by caspase-1, which is recruited to the NLRP3 inflammasome complex by binding ASC. In addition, NLRP3 activation triggers an inflammatory caspase-1 dependent death process termed pyroptosis (Miao et al., 2011). While IL-1 $\beta$  processing and release require caspase-1 catalytic activity, pyroptosis can be mediated by uncleaved caspase-1 following activation of NLRC4 or AIM2 inflammasomes (Broz et al., 2010).

While the exact mechanism of NLRP3 inflammasome activation has remained elusive, a variety of danger associated molecular patterns and pathogen associated molecular patterns, including ATP, nigericin, pore forming toxins, silica crystals, and alum have been shown to trigger inflammation through NLRP3 activation (Bauernfeind et al., 2011b). The chemical diversity of these stimuli suggests that they may induce a common cellular stress response rather than binding directly to the NLRP3 inflammasome. Potassium efflux from cells is required for NLRP3 activation by almost all stimuli examined, suggesting that compromised membrane integrity is a common feature of this pathway (Petrilli et al., 2007).

Several reports attributed the activation of the NLRP3 inflammasome to the leakage of lysosomal contents into the cytosol following phagocytosis of particulate stimuli that could damage their integrity (Halle et al., 2008; Hornung et al., 2008). Using inhibitors that blocked inflammasome activation, it was suggested that cathepsin B might be responsible for proteolytically activating NLRP3 inflammasome (Hornung et al., 2008). This process, characterized by the loss of lysosomal contents and acidity, has been termed lysosomal

membrane permeability (LMP) (Newman et al., 2009). There remains uncertainty about the generality of this model since not all NLRP3 activators are phagocytosed and disrupt lysosomes directly. Further, it has been suggested that the cathepsin B inhibitor used, CA-074 Me, may not be specific, as RNAi knockdown and use of cells from cathepsin B<sup>-/-</sup> mice did not recapitulate CA-074 Me results (Dostert et al., 2009; Newman et al., 2009). Alternatively, release of reactive oxygen species (ROS) into the cytosol during LMP could trigger NLRP3 activation, suggested by the observation that NLRP3 inflammasome can be activated by addition of superoxide to macrophage cultures (Zhou et al., 2010). However, inhibition of lysosomal ROS generation with Nox1-4 knockout mice did not show a corresponding decrease in NLRP3 inflammasome activation (Latz, 2010; Zhou et al., 2010).

A role for mitochondria as another potential source of ROS has also been explored (Sorbara and Girardin, 2011; Zhou et al., 2010). Mitochondria are sensitive to cellular stress, and respond through depolarization of the mitochondrial membrane, ROS release, a decrease in mitochondrial ATP, and reversible opening of the mitochondrial permeability transition pore. A later marker of mitochondrial damage, mitochondria membrane permeability (MMP), can result in the release of additional mitochondrial contents, including cytochrome c, and eventual cell death (Boya et al., 2003a; Green and Kroemer, 2004).

Recent reports showing that mitochondrial DNA (mtDNA) can activate the NLRP3 inflammasome have further supported a role for mitochondria in inflammasome activation (Martinon, 2012). Inhibition of mitophagy promotes the accumulation of dysfunctional mitochondria in cells, and their eventual breakdown was suggested to increase levels of mtDNA in the cytosol (Nakahira et al., 2011). A second study showed directly that oxidized mtDNA can activate NLRP3 inflammasome (Shimada et al., 2012).

Here, we present evidence that NLRP3 inflammasome activation occurs prior to both LMP and MMP. Using a combination of live cell imaging and biochemical approaches, we demonstrate that a prototypical NLRP3 stimulus, the potassium ionophore nigericin, exerts both NLRP3 independent and dependent effects on macrophages that result in cytokine release and cell death. We present a kinetic model integrating the roles of mitochondria and lysosomes in this process.

## 2.4 MATERIALS AND METHODS

*BMDM preparation-* Bone marrow was harvested from the tibiae and femora of WT or NLRP3<sup>-/-</sup> B6 mice (gifts from Dr. Lisa Borghesi, Dr. Olivera Finn, and Dr. Timothy Billiar). Bone marrow cells were grown in DMEM (Cellgro) supplemented with 20% fetal bovine serum (FBS) (Gemcell), 2 mM L-glutamine (Cellgro), 500 U penicillin/500 µg streptomycin (Lonza), 1 mM sodium pyruvate (MP Biomedical), and 30% L929 cell (American Type Culture Collection CCL-1) supernatant for 8-25 days. Media was replaced after four days and cells passaged every four days after day 7. For experiments, cells were harvested with Cellstripper (Cellgro) and plated in IMDM (HyClone) supplemented with 10% FBS, 2 mM L-glutamine, 500 U penicillin/500 µg streptomycin (cIMDM). L929 cell supernatants were generated by culturing L929 cells for six days in DMEM supplemented with 10% FBS 2 mM L-glutamine, 500 U penicillin/500 µg streptomycin, 1 mM sodium pyruvate and 1x non-essential amino acids (MP Biomedical).

*TLO Preparation-* Tetanolysin O (Enzo) was reduced in DTT. Cells were washed to remove serum prior to the addition of TLO. 100 ng/ml TLO was added in serum free media to appropriate dishes and incubated at 37°C for the duration of imaging as indicated.

*MTT assay-* WT BMDM were plated one day before the experiment at 500,000 cells/well in a 12 well tissue culture plate (Costar) in cIMDM, as described above. Cells were incubated for 4 hours with 1 µg/ml LPS from *E. coli* 026:B6 (Sigma L2654). 50 mM KCl, 100 µM or 500 µM MitoTEMPO (Enzo) were added during the last 30 minutes of LPS treatment and then re-added when media was removed and 1 ml fresh phenol red-free cIMDM (Gibco) was added to the cells. 20 µM nigericin sodium salt (Sigma) or 3 mM ATP (Sigma) was added to the cells and incubated at 37°C for 5-30 minutes. After supernatants were removed, cells were washed once with 1x DPBS (Cellgro) and analyzed using the Vybrant® MTT cell proliferation assay kit (Life Technologies) according to the manufacturer's instructions and then assayed in duplicate for OD using a PowerWave XS microplate spectrophotometer (BioTek). The percentage of MTT unconverted to formazan in the sample was calculated as  $100 - (\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{blank}}) / (\text{mean OD}_{\text{untreated control}} - \text{mean OD}_{\text{blank}}) \times 100$ .

*LDH Release Assay-* WT BMDM were treated as described above for the MTT assay. 1% Triton X-100 (Fisher Scientific) was incubated with cells for five minutes as a positive control for complete cell lysis. Supernatants were removed and assayed in duplicate using an LDH cytotoxicity kit (Cayman Chemical) for OD using a PowerWave XS microplate spectrophotometer. Percent LDH release was calculated as  $(\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{blank}}) / (\text{mean OD}_{\text{positive control}} - \text{mean OD}_{\text{blank}}) \times 100$ .

*Quantification of ATP remaining in cell-* WT or NLRP3<sup>-/-</sup> BMDM were treated as described for the LDH assay. Supernatants were removed. Using CellTiter-Glo Luminescent Cell

Viability Assay (Promega), ATP levels remaining in the cell lysates were determined using a luminometer according to the kit instructions. Results were recorded as a percentage of ATP remaining in cells based on untreated control cells.

*Seahorse Assay-* WT or NLRP3<sup>-/-</sup> BMDM were plated the night before at 500,000 cells/well in a Seahorse 24 well V7 microplate. Cells were LPS primed at 1 µg/ml for four hours. Media was removed from wells and cells were incubated in buffered growth media for an hour after pre-treatment with nitrite. 20 µM nigericin was added into wells in unbuffered media prior to inserting plate into Seahorse XF24 Extracellular Flux analyzer (Seahorse Bioscience). After 21.5 minutes of equilibration, media was injected as a control, oligomycin (20 µM) was injected into each well at 48 minutes, followed by FCCP (75 µM) at 73 minutes, and rotenone (20 µM) at 99 minutes. OCR was recorded as pMoles/min. Averages of three wells were taken per data point.

*Live cell microscopy-* WT or NLRP3<sup>-/-</sup> BMDM were plated at 250,000 cells/dish in 35 mm collagen coated glass bottom culture dishes (MatTek) the night before the experiment in cIMDM. Media was removed and cells were LPS primed (1 µg/ml) for three to four hours in fresh media. Inhibitors were added during the last 30 minutes of LPS priming at 37°C and were added back to the dish in fresh media before imaging, following washing out LPS containing media. YVAD was added at 100 µM, CA-O74 Me (Calbiochem) was added at 100 µM. 2µM MitoTracker Red CM-H2XRos (Invitrogen), 10 µM TMRM, or 2 µM LysoTracker Green DND-26 (Invitrogen) were added during the last 30 minutes of LPS priming. 6.5 µM JC-1 was added during the last 20 minutes of LPS priming. 5 µM MitoSOX (Invitrogen) was added 10 minutes prior to imaging at 37°C. 10 µl/ml pSIVA (Imgenex) was added in 1 ml of complete IMDM two frames after the initiation of imaging after 30 minutes pre-incubation. Dishes were imaged on

Nikon A1 inverted microscope with a Plan Fluor 40x objective with an 1.30 NA or Plan Apo 60x objective with an 1.40 NA, Photometrics Coolsnap HQ2 or Andor™ Technology iXon<sup>EM</sup> + camera, NikonPiezo driven XYZ stage, and Tokai Hit Environmental chamber. For MitoTracker, LysoTracker, JC-1, TMRM, and pSIVA, images were captured every 30 seconds for one hour. MitSOX was imaged every minute. 20  $\mu$ M nigericin was added 2 minutes into imaging in 1ml of media. Videos were analyzed using Elements (Nikon) and Microsoft Excel.

*IL-1 $\beta$  ELISA*- WT or NLRP3<sup>-/-</sup> BMDM were plated at 500,000 cells/well in a 12 well tissue culture plate one night before. Cells were LPS-primed for four hours and treated as indicated. 100 $\mu$ M A740003 (Tocris) or 50 or 100 $\mu$ M 5-BDBD (Tocris) were added as indicated. Supernatants were analyzed by IL-1 $\beta$  ELISA as follows. A 96 well plate was coated in 4  $\mu$ g/ml of purified mouse anti-IL-1 $\beta$  capture antibody (eBioscience) at 4°C overnight. The wells were blocked in 1% filtered BSA (Sigma) for two hours at room temperature and then samples were added to the wells for two hours at room temperature. Biotin conjugated anti-IL-1 $\beta$  mouse detection antibody (eBioscience) was added at 6  $\mu$ g/ml for one hour at room temperature. Avidin-HRP (Biolegend) was added to wells at 1:5000 for 30 minutes at room temperature. Equal volumes of TMB (Biolegend) reagent A and B were mixed and then 100  $\mu$ l was added to each well for no more than 20 minutes at room temperature. 50  $\mu$ l of 1M sulfuric acid was added to wells to stop the reaction. OD values were read at 450 nm with 570 nm background subtraction using BioTek® PowerWave XS Microplate Spectrophotometer and Gen5™ Data Analysis Software.

*IL-1 $\beta$  Western Blot*- Lysates and supernatants were collected from  $2.0 \times 10^6$  BMDM after treating cells as indicated in serum-free IMDM. Lysates were prepared using NP-40 lysis buffer (Boston BioProducts) containing a protease inhibitor cocktail (Calbiochem). Supernatants

were TCA precipitated and both lysates and supernatants were analyzed for IL- $\beta$  via Western blot as previously described (Chu et al., 2009). Briefly, the entire precipitated supernatant or 50  $\mu$ g of protein of lysate was loaded onto an 11% polyacrylamide gel. Samples were run using a Labnet Power Station 300 and then transferred onto a PVDF membrane (Thermo) using the iBlot (Invitrogen) system according to the manufacturer's instructions. Western blotting was performed using the SNAPid system (Millipore) according to the manufacturer's directions. Mouse antibodies against NLRP3 (Enzo), ASC (Millipore clone 2E1-7), or IL-1 $\beta$  (3ZD clone from National Cancer Institute) were used as primary antibodies and goat antibodies against mouse conjugated to HRP (Santa Cruz) was used as a secondary antibody at a 1:1666 dilution. Enhanced chemiluminescence reagent (Santa Cruz) was added before imaging on a Kodak Image Station 4000MM (Molecular Imaging Systems).

*Statistical analysis-* All data sets were analyzed via a student's two-tailed t-test unless otherwise noted using Graphpad software. P values were as indicated and were considered significant if  $P < 0.05$ . Significance verified via ANOVA.

## 2.5 RESULTS

### 2.5.1 NLRP3 inflammasome activation increases sensitivity of bone marrow derived macrophages to membrane damage

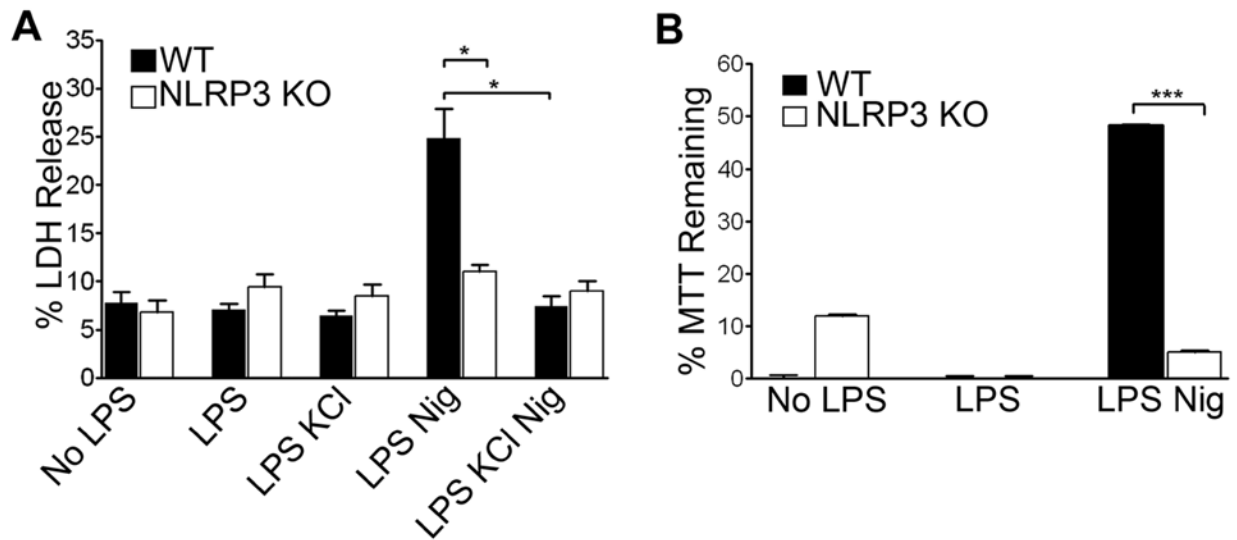
NLRP3 inflammasome is present in a variety of immune cells, including macrophages. Due to the robustness of NLRP3 activation in murine bone marrow derived macrophages (BMDM), this

system has frequently been employed to examine NLRP3 inflammasome activation and function. BMDM were primed with LPS for three to four hours to up-regulate inflammasome components as well as induce the translation of pro-IL-1 $\beta$ , a cytokine that is processed into its active form during NLRP3 inflammasome dependent caspase-1 activation. Following priming, BMDM from wild type C57/BL6 mice were treated with the potassium ionophore nigericin, a prototypical activator of the NLRP3 inflammasome.

To test membrane permeability, lactate dehydrogenase (LDH) was measured in the culture supernatants. Following nigericin treatment, LDH levels were increased in the supernatants of LPS primed wild type BMDM but not LPS primed NLRP3<sup>-/-</sup> cells, indicating that the cell membrane integrity had been compromised in the former (Figure 2-1A). Pre-treatment of WT BMDM with 50 mM KCl to block potassium efflux significantly inhibited release of LDH. Non-LPS primed or BMDM treated with LPS alone did not release LDH above background levels.

We next used a second assay to measure cell viability, the conversion of MTT to formazan, which is carried out by mitochondrial reductases. LPS-primed WT and NLRP3<sup>-/-</sup> BMDM also differed significantly in MTT reduction after nigericin exposure (Figure 2-1B). Together, these results demonstrate that the NLRP3 inflammasome mediates membrane damage.





**Figure 2-1: NLRP3 dependent membrane damage**

(A) LPS primed or unprimed WT and NLRP3<sup>-/-</sup> BMDM were treated with nigericin for thirty minutes with or without KCl. LDH release was recorded as the percent released into the supernatant by five minutes of Triton treatment. LDH assay was allowed to develop for two hours and readings were taken as an average of two wells. Error bars represent mean  $\pm$  s.d. (n=3, \*, P<0.01; LPS Nig WT vs KO: P=0.009; WT LPS Nig vs WT LPS Nig KCl: P=0.004). (B) Conversion of MTT reagent added to cell lysates was measured as absorbance of the colored product formazan. Data were acquired as the percent of absorbance of each well/absorbance of Triton-X treated control cells and graphed as MTT reagent remaining unconverted by mitochondrial reductases. Readings were taken as an average of two wells. Error bars represent mean  $\pm$  s.d. (n=4, \*\*\*, P<0.0001).

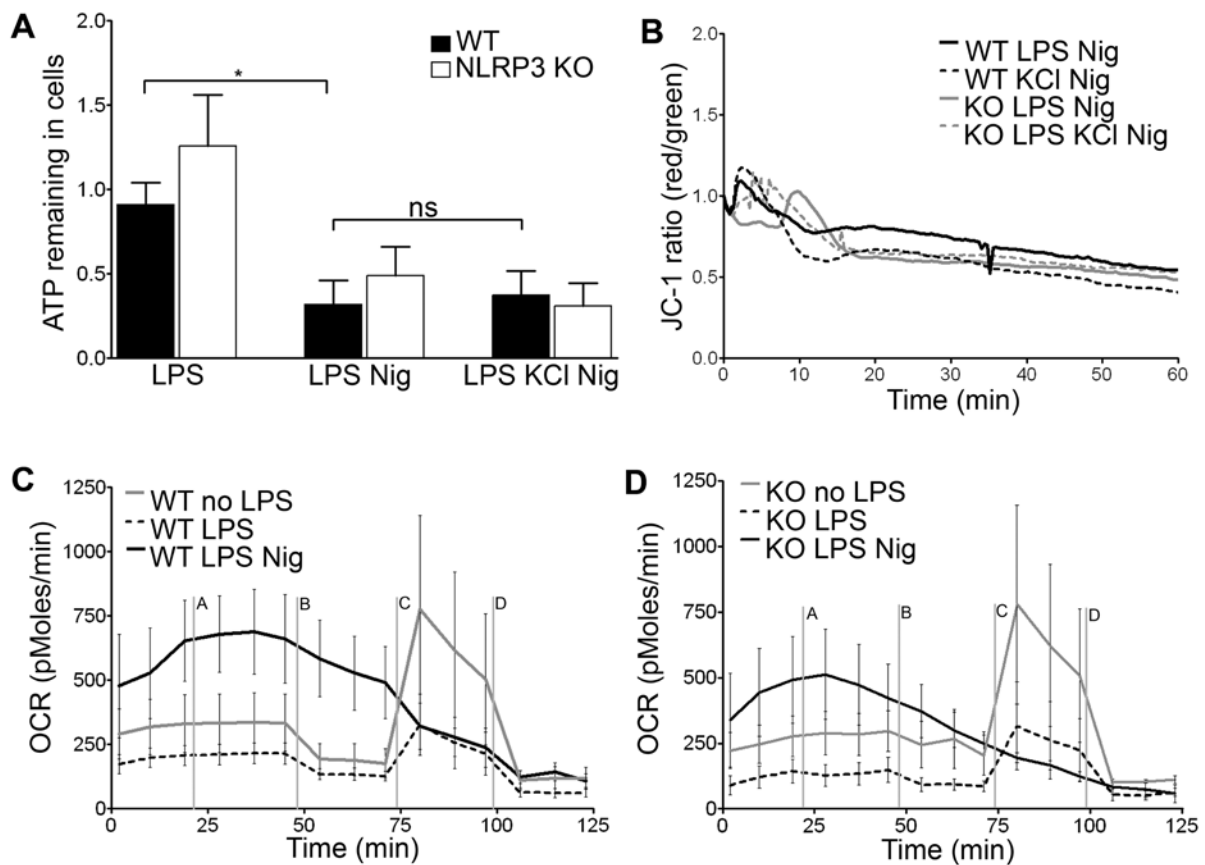
### 2.5.2 Nigericin alters mitochondrial function independently of NLRP3

Since mitochondria have been implicated in NLRP3 inflammasome activation, we wanted to explore how inflammasome activating stimuli impact mitochondrial function (Zhou et al., 2010). To accomplish this, we measured ATP levels in LPS primed BMDM following nigericin

exposure. Both WT and NLRP3<sup>-/-</sup> nigericin treated BMDM contained reduced amounts of ATP compared to non-nigericin treated cells, suggesting either decreased synthesis, increased consumption, or release of ATP into the culture supernatant (Figure 2-2A). ATP levels in nigericin treated cells were not preserved by co-exposure to KCl, consistent with a NLRP3-independent event.

To measure mitochondrial depolarization in response to nigericin, the ratiometric dye JC-1 was used in live cell imaging. WT and NLRP3<sup>-/-</sup> BMDM both depolarized in response to nigericin following a rapid but slight hyperpolarization (Figure 2-2B). Mitochondrial depolarization occurred independently of NLRP3 and was insensitive to inhibition by KCl.

To further characterize nigericin-induced alterations in mitochondrial function, we used the Seahorse assay to measure oxygen consumption rate (OCR) in WT and NLRP3<sup>-/-</sup> BMDM. Basal OCR levels in non-primed WT and NLRP3<sup>-/-</sup> BMDM were comparable, while both showed reduced OCR levels four hours after LPS priming. Nigericin rapidly increased OCR levels in both LPS primed WT and LPS primed NLRP3<sup>-/-</sup> BMDM, indicating a stress response in mitochondrial respiration (Figures 2-2C and 2-2D). Following nigericin exposure, cells from both cell types were unable to respond to oligomycin or FCCP, which respectively inhibit ATP synthesis and uncouple electron transport. In contrast, LPS primed WT or NLRP3<sup>-/-</sup> BMDM decreased OCR in response to oligomycin, followed by increased OCR in response to FCCP, which could be blocked by rotenone, a complex I inhibitor. These data show that LPS priming and nigericin each disrupt mitochondrial respiration in BMDM independently of NLRP3, with more severe dysfunction occurring after nigericin treatment. Taken together, these data demonstrate that nigericin reduces OCR, induces mitochondrial depolarization, and diminishes cellular ATP levels independently of NLRP3 in BMDM.



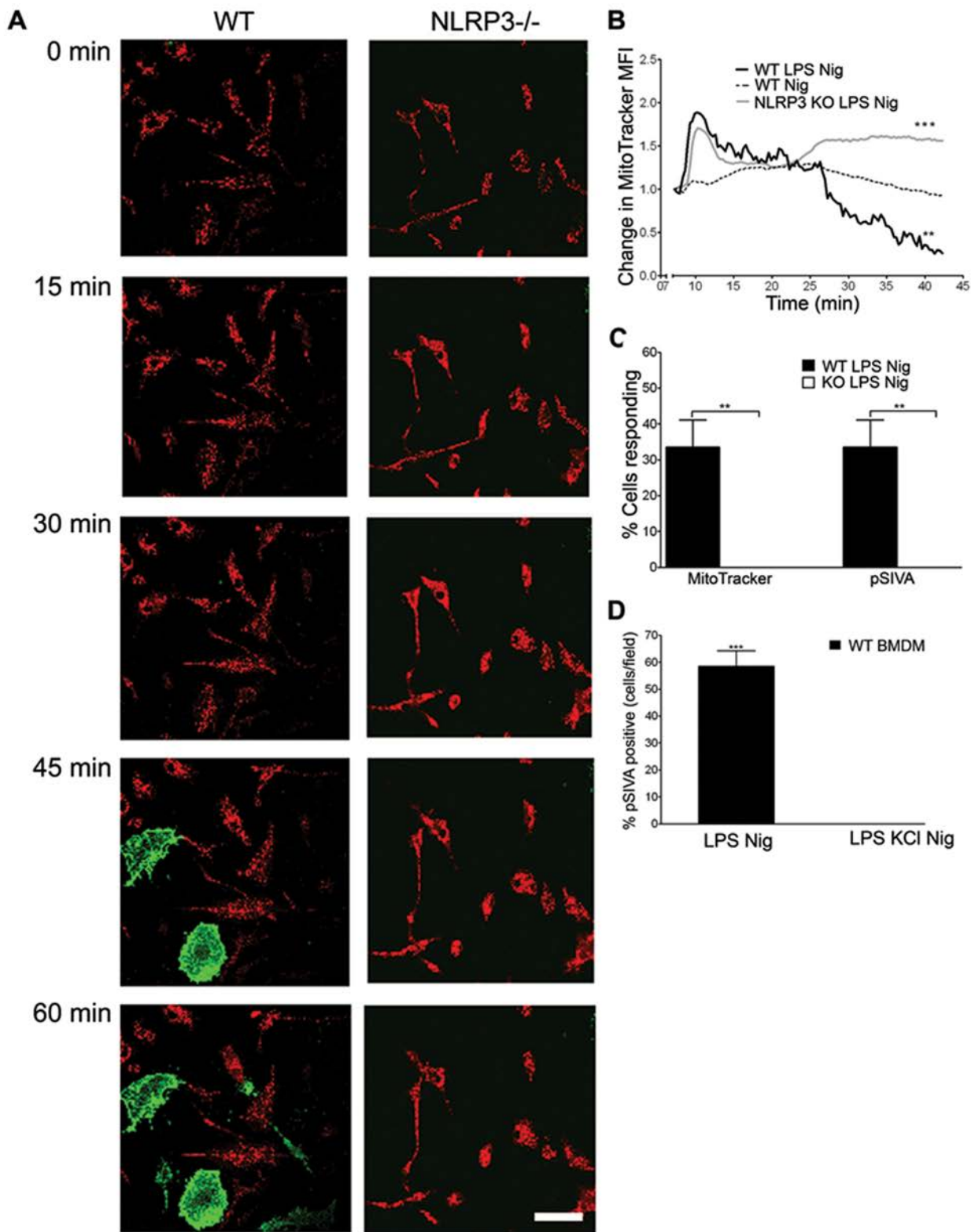
**Figure 2-2: Loss of ATP, decrease in OCR, and depolarization are NLRP3 independent**

WT or NLRP3<sup>-/-</sup> BMDM were LPS primed and (A) treated with nigericin for thirty minutes with or without KCl. Supernatants were collected and ATP levels measured. Error bars represent ± s.d. (n≥6, \*, P=0.0103; ns, p=0.7941) (B) Live cell wide field microscopy was used to determine the rate of mitochondrial depolarization with JC-1. Cells were LPS primed and nigericin treated. Ratio of red/green signal was obtained over 1 hour of imaging. Data comprise at least 2 separate experiments and was normalized to initial signal. WT BMDM(C) or NLRP3<sup>-/-</sup> BMDM (D) were treated with nigericin immediately before inserting plate in Seahorse machine. OCR was recorded as an average of 3-4 wells. Media (line a), oligomycin (b), FCCP (c), and rotenone (d), were added as indicated. Error bars represent ± s.d.(n=4, at 45 min, ns, p=0.3099).

### 2.5.3 Loss of mitochondrial membrane integrity and cell death are NLRP3 dependent

Since WT and NLRP3<sup>-/-</sup> BMDM differ greatly in their ability to convert MTT through mitochondrial reductases (Fig 2-1B), we further probed potential effects of NLRP3 on mitochondria using MitoTracker Red, to measure membrane integrity. Using live cell microscopy, we found that nigericin treated, LPS primed WT and NLRP3<sup>-/-</sup> BMDM showed distinct differences in mitochondrial integrity (Figures 2-3A and 2-3B). Approximately 30% of WT BMDM exhibited complete loss of mitochondrial integrity, while no loss was detected in NLRP3<sup>-/-</sup> BMDM in one hour (Figure 2-3C). In non-primed BMDM, no loss of mitochondrial integrity was seen (Figure 2-3B). Loss of mitochondrial membrane integrity correlated with exposure of phosphatidylserine (PS) at the plasma membrane (Figures 2-3A and 2-3C). PS exposure was inhibited by the addition of KCl (Figure 2-3D). These data demonstrate that NLRP3 inflammasome increases sensitivity of BMDM to loss of mitochondrial membrane integrity and subsequent cell death.

We investigated mtDNA localization in cells using PicoGreen, a dsDNA specific dye which labels mitochondria and nuclei (Ashley et al., 2005). We observed a decrease in mitochondrial PicoGreen staining in LPS-primed WT BMDM, but not in NLRP3<sup>-/-</sup> BMDM after 1h exposure to nigericin (data not shown). Loss of mtDNA in WT cells was prevented by KCl (data not shown), and coincided with loss of MitoTracker Red.



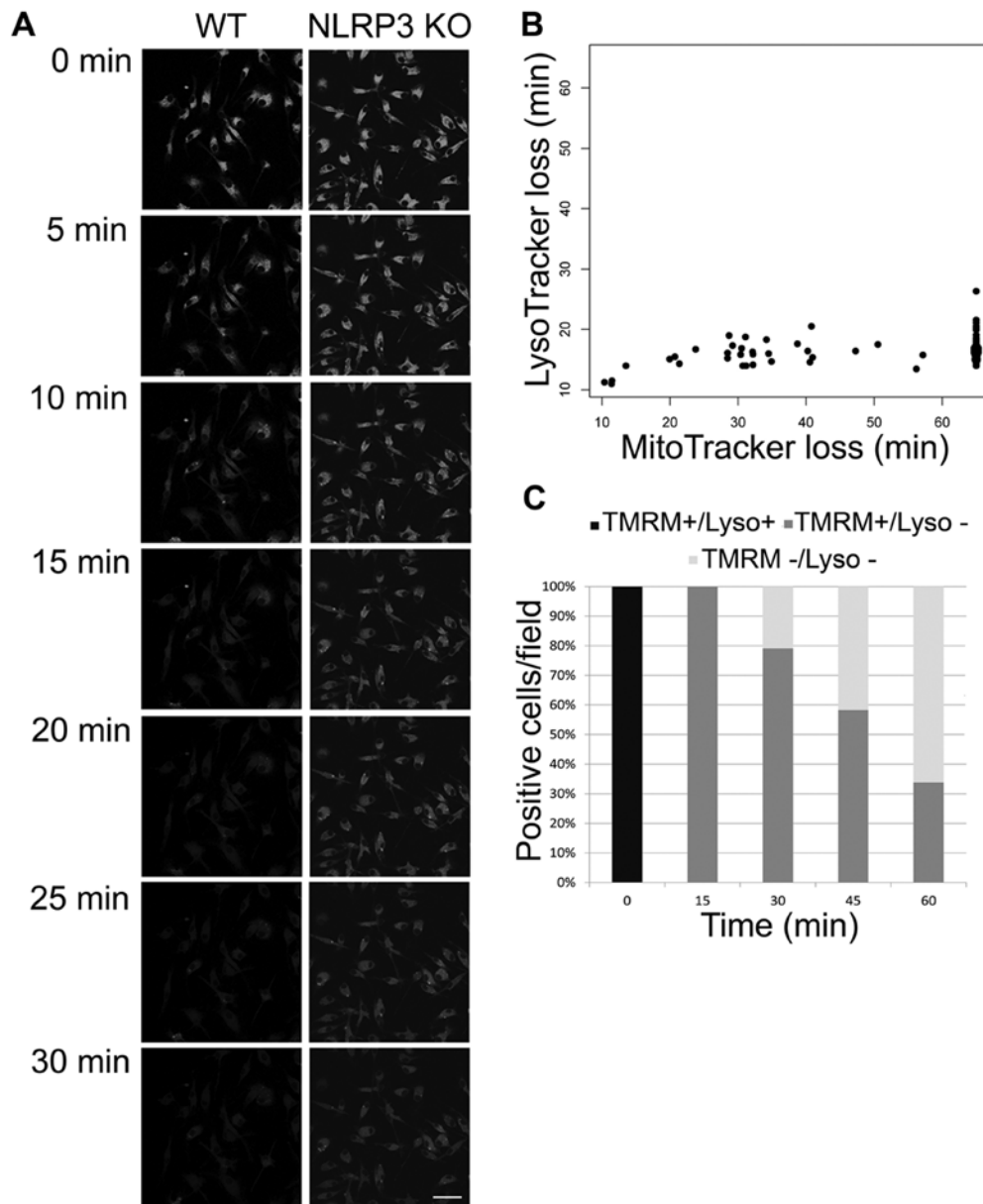
### **Figure 2-3: Loss of mitochondrial permeability and cell death are NLRP3 dependent**

(A) WT and NLRP3<sup>-/-</sup> BMDM were LPS primed and treated with 20  $\mu$ M nigericin. Cells were stained with MitoTracker red (red) and pSIVA (green), imaged for one hour with a confocal live cell microscope. Images from one representative experiment shown. Scale bar= 50  $\mu$ m (B) Loss of MitoTracker red signal over time was recorded via live cell microscopy. Data was normalized to initial signal with background subtraction. (n=6; at 40 min. \*\*\*, p=0.0001; \*\*, p=0.0015) (C) Percentage of cells/field that lost MitoTracker signal or gained pSIVA signal over 1 hour of confocal live cell imaging. Error bars represent  $\pm$  s.d. (MitoTracker n=4; \*\*, p=0.002; pSIVA, n=3; \*\*, p=0.002) (D) Cells were treated with or without 50 mM KCl and imaged for one hour on a confocal live cell microscope. Percent of cells/field that were pSIVA positive at 60 min recorded. Error bars represent  $\pm$  s.d. (n=3; \*\*\*, p=0.0001).

#### **2.5.4 Lysosomal membrane permeabilization precedes mitochondrial integrity breakdown during NLRP3 activation**

A previously proposed model for NLRP3 activation suggested that following phagocytic uptake of indigestible particles known to trigger IL-1 $\beta$  secretion, rupture of phagolysosomes would release proteases into the cytosol leading to assembly and activation of the NLRP3 inflammasome (Hornung et al., 2008). We evaluated one aspect of this model using live cell imaging. Lysosomes labeled with LysoTracker Green lose fluorescence within 15 min after exposure to nigericin in LPS-primed WT cells (Figure 2-4A), consistent with either loss of lysosomal acidity or a loss of lysosomal membrane integrity in a process termed LMP. Labeled lysosomes in NLRP3<sup>-/-</sup> BMDM however retained fluorescence after nigericin treatment, demonstrating that LMP is NLRP3 dependent. A second NLRP3 inflammasome activator, the pore forming toxin tetanolysin O (TLO), also induced LMP selectively in WT BMDM (Figure 2-5A). LMP always preceded MMP, occurring within 15 minutes of exposure to nigericin, while

MMP typically required between 30-60 min (Figure 2-4B). In addition, all WT BMDM exposed to nigericin underwent LMP, while on average no more than 60-70% underwent MMP within 60 min (Figure 2-4C). LMP was blocked in WT BMDM by KCl, consistent with NLRP3 dependence of the process (Figure 2-5B). Our results demonstrate that NLRP3 inflammasome activation results in loss of membrane integrity in lysosomes and mitochondria in kinetically distinct events.

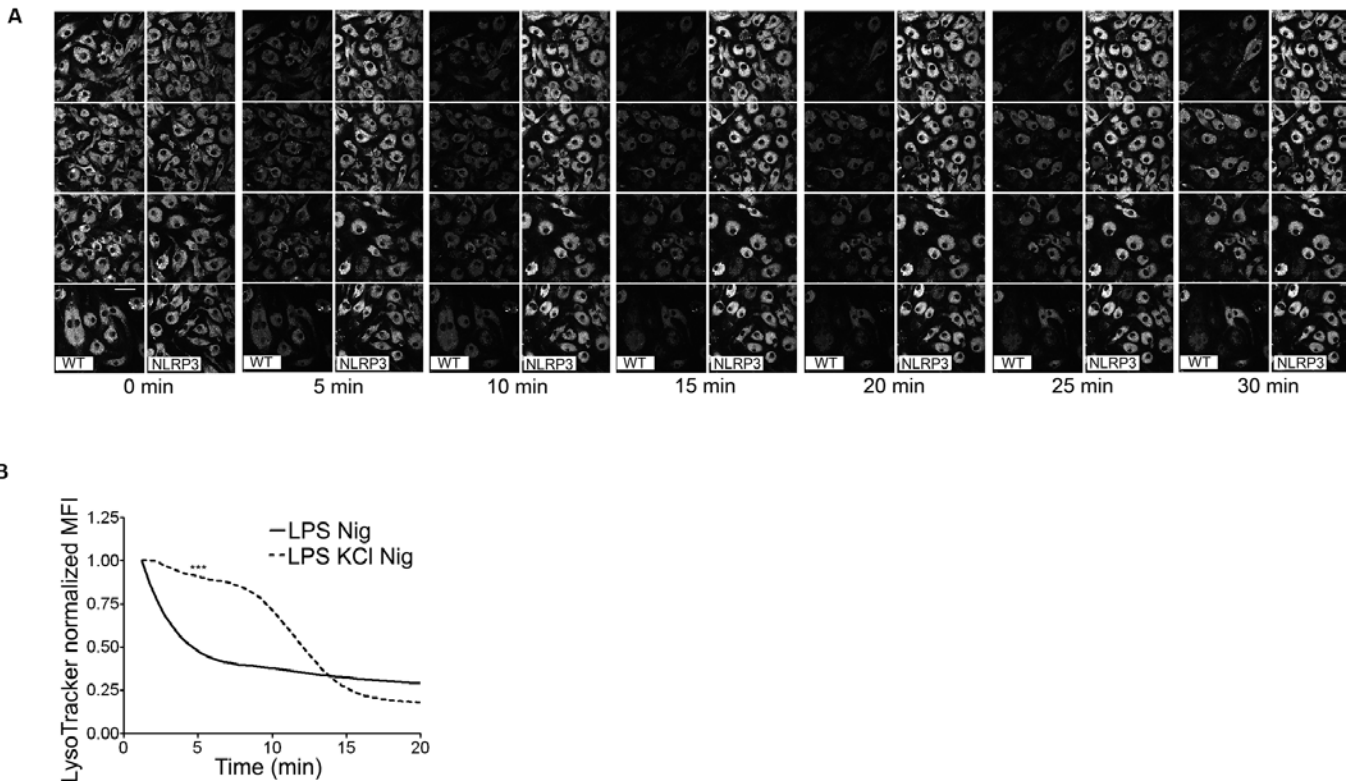


**Figure 2-4: Loss of lysosomal membrane permeability is NLRP3 dependent**

(A) WT and NLRP3<sup>-/-</sup> BMDM were LPS primed, stained with LysoTracker Green and imaged via live cell confocal microscopy with 20  $\mu$ M nigericin treatment for 30 min. Data from one representative experiment shown. Scale bar= 50  $\mu$ m (B) WT BMDM were LPS primed, treated with nigericin, and stained with MitoTracker Red and LysoTracker Green. Cells were imaged for one hour after nigericin addition via live cell microscopy. Data were



recorded as time to loss of signal, each dot represents one cell. Cells that did not lose MitoTracker Red were recorded as data points beyond 60 min imaging period. Data from five fields are displayed. (C) WT BMDM were LPS primed and nigericin treated, stained with TMRM and LysoTracker Green, and imaged with live cell microscopy for 1 hr. Percent of cells/field staining positive for each of the two dyes was recorded. n=9 fields.

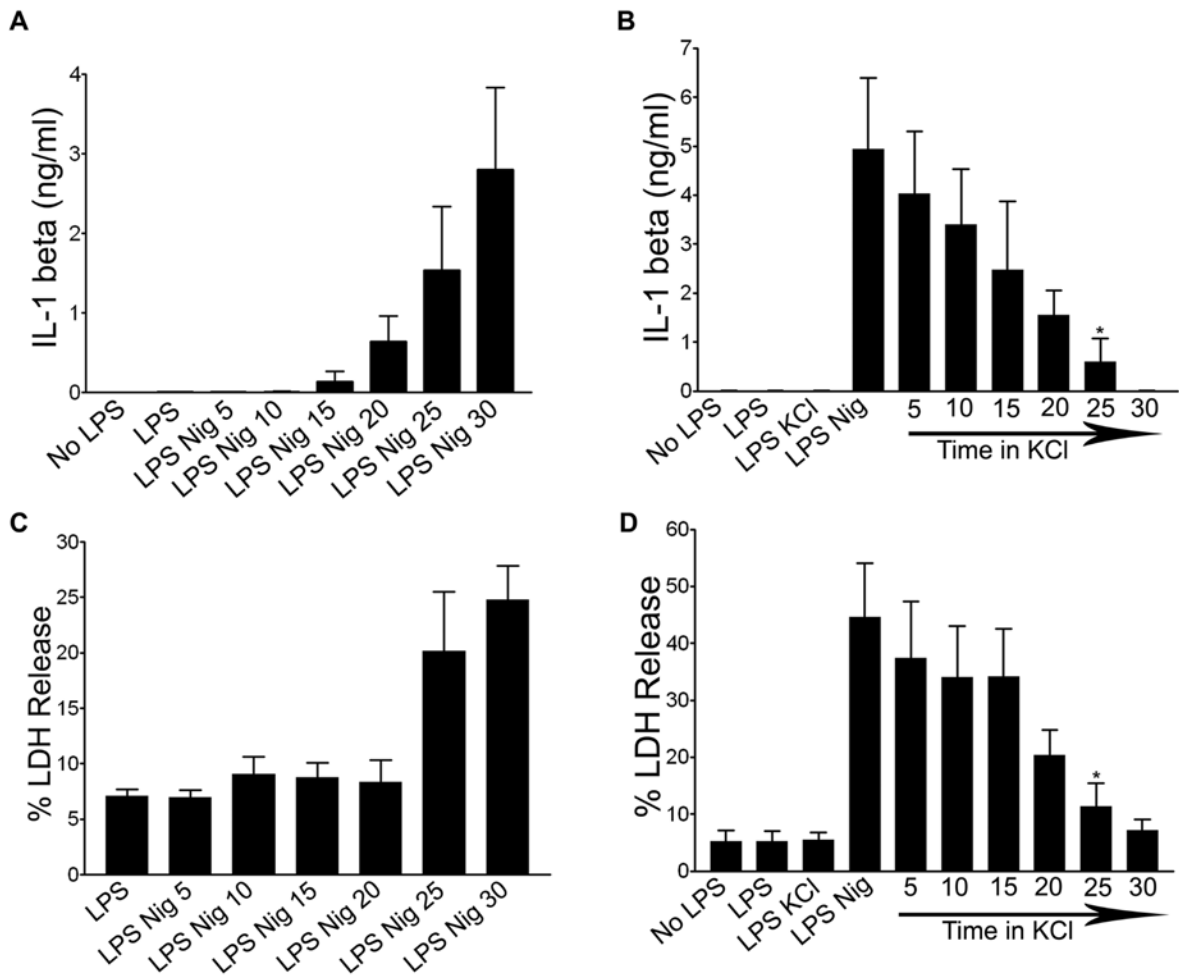


**Figure 2-5: TLO induced LMP is NLRP3 dependent and KCl delays LMP in WT BMDM**

(A) WT or NLRP3<sup>-/-</sup> BMDM labeled with LysoTracker Green were treated with 100 ng/ml tetanolysin O (TLO) following LPS priming. Live cell microscopy was used to detect loss of LysoTracker Green signal following treatment for 30 minutes. Data are displayed as four representative fields/cell type. Scale bar= 50  $\mu$ m. (B) WT BMDM were LPS primed and treated with 20  $\mu$ M nigericin with or without 50 mM KCl. Cells were labeled with LysoTracker Green and imaged via wide field live cell microscopy. Nigericin was added after 2 min. LysoTracker MFI was normalized to initial signal after background subtraction. Data from five fields (LPS Nig) or 10 fields (LPS KCl Nig) are shown. (5 min, \*\*\*, P<0.0001).

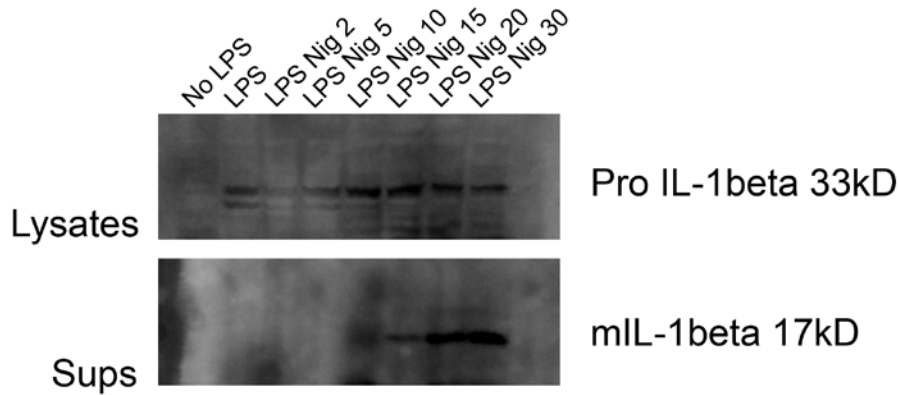
### **2.5.5 IL-1 $\beta$ processing and secretion kinetically precedes loss of mitochondrial membrane integrity**

A major function of the NLRP3 inflammasome in immunity is to regulate secretion of a family of non-conventionally secreted cytokines, including IL-1 $\beta$ . To probe the role of mitochondria and lysosomes in cytokine secretion, we performed kinetic analysis of processed IL-1 $\beta$  secretion. Processed IL-1 $\beta$  was detected in the supernatants of LPS primed WT BMDM 15 minutes after nigericin exposure, and levels continued to increase over thirty minutes of nigericin exposure (Figure 2-6A). Western blotting was used to confirm that released IL-1 $\beta$  was the cleaved biologically active form, not unprocessed precursor (Figure 2-7). IL-1 $\beta$  release was significantly decreased by addition of KCl five minutes after nigericin exposure, with progressively less inhibition occurring with KCl added at later times within the 30 min nigericin incubation period (Figure 2-6B). Since LPS primed, nigericin exposed BMDM lose mitochondrial integrity and expose plasma membrane PS by about 30 minutes (Figures 2-3 and 2-4), this suggests that IL-1 $\beta$  secretion precedes cell death and MMP, and correlates kinetically with LMP. LDH release occurred with nearly identical kinetics to IL-1 $\beta$  secretion and was also inhibited by KCl, consistent with compromised plasma membrane integrity before cell death (Figures 2-6 C-D).



**Figure 2-6: IL-1 beta processing and secretion is kinetically distinct from MMP**

WT BMDM were LPS primed or unprimed and (A) treated with 20  $\mu$ M nigericin for 5-30 minutes. Supernatants were collected and analyzed for IL-1 $\beta$  using ELISA. Error bars represent  $\pm$  s.d. n=3 (B) WT BMDM were treated with nigericin for thirty minutes. 50 mM KCl was added after 0-25 minutes following nigericin treatment. Supernatants were collected and analyzed via IL-1 $\beta$  ELISA. Error bars represent  $\pm$  s.d. n $\geq$ 3 (\*, P=0.0294 for LPS Nig KCl 25 vs LPS Nig). (C) WT BMDM were LPS primed and treated with nigericin for 5-30 minutes or (D) were LPS primed and then had 50 mM KCl added 0-25 minutes following nigericin treatment. Supernatants were collected for LDH release analysis. Data shown as percentage of LDH release by Triton-X 100 treated control cells. Error bars represent  $\pm$  s.d. n=4 (\*, P=0.0475 for LPS Nig KCl 25 vs LPS Nig).



**Figure 2-7: Processed IL-1 $\beta$  is detected after 15 min nigericin stimulation**

WT BMDM were LPS primed or left unprimed and then treated with 20  $\mu$ M nigericin for 2-30 minutes. Lysates and precipitated supernatants were blotted for mature IL-1 $\beta$  (17kD) or pro IL-1 $\beta$  (33kD).

### 2.5.6 IL-1 $\beta$ secretion distinct from organelle damage and cell death

To characterize the mechanisms underlying MMP, LMP, and IL-1 $\beta$  processing and secretion, we examined the requirement for caspase-1 in each process. Ac-YVAD-cmk (YVAD), a caspase-1 inhibitor, did not prevent the loss of lysosomal membrane integrity or mitochondrial membrane integrity in WT BMDM treated with nigericin (Figure 2-8A). Furthermore, YVAD pre-treatment blocked processing and secretion of IL-1 $\beta$  but did not significantly inhibit LDH release from WT BMDM (Figures 2-8B and 2-8C). While caspase-1 activity is required for the processing and secretion of mature IL-1 $\beta$ , it does not appear to be required in its active form to trigger LMP, MMP, or cell death.

We and others have shown the cathepsin B inhibitor, CA-074 Me, inhibits secretion of processed IL-1 $\beta$  (Chu et al., 2009; Hornung et al., 2008). Cathepsin B is a lysosomal protease that is likely released during LMP. Since processing and secretion of IL-1 $\beta$  correlates kinetically

with LMP (Figures 2-4 and 2-6), we tested whether cathepsin B participates in MMP. CA-074 Me did not inhibit LMP or MMP induced by nigericin in LPS primed BMDM (Figure 2-9). These results show that the role of NLRP3 inflammasome in IL-1 $\beta$  processing and secretion is distinct from effects on lysosomal and mitochondrial membrane integrity in its dependence on caspase-1 and cathepsin B.

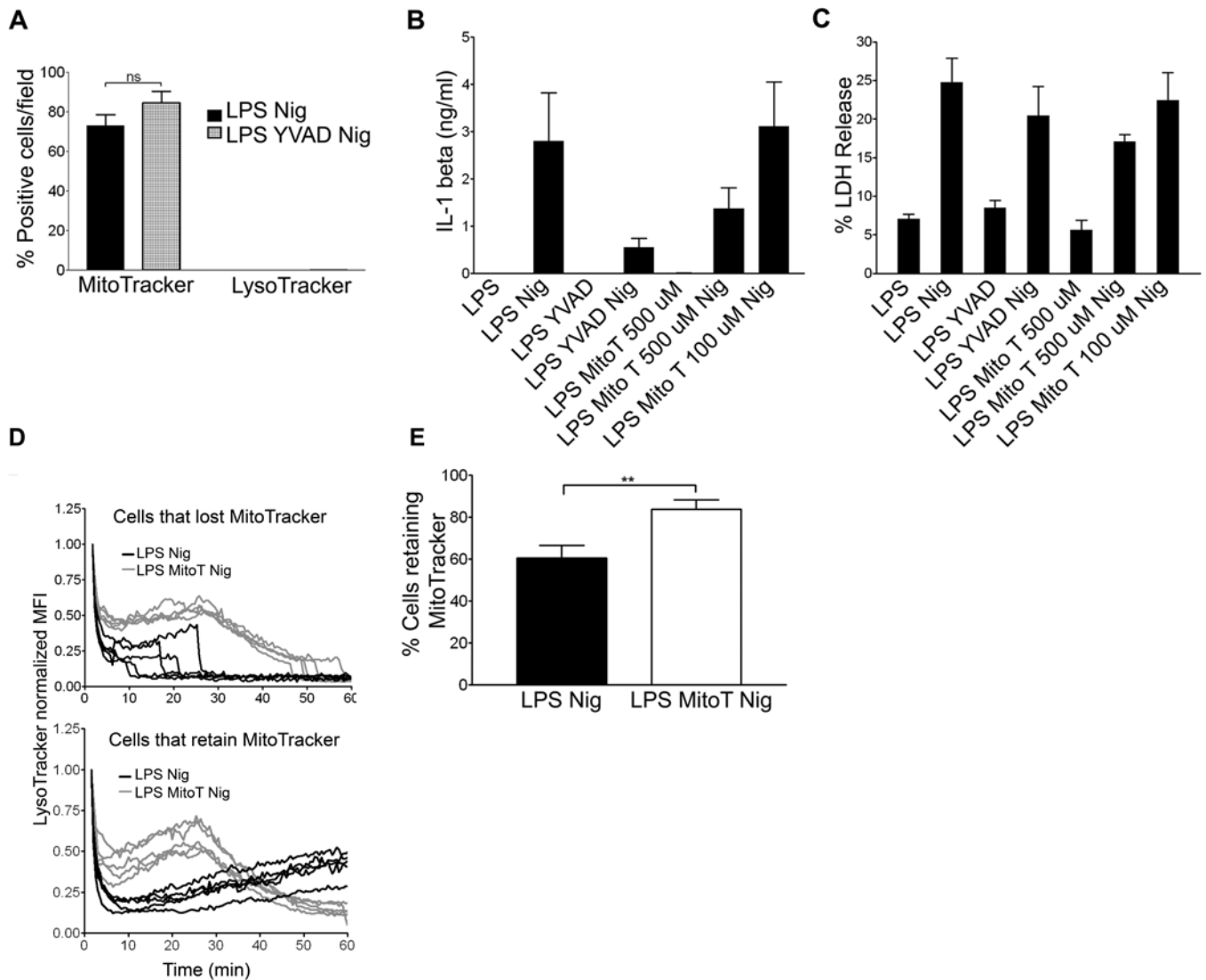
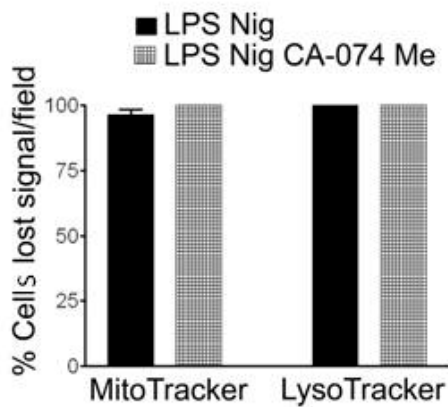


Figure 2-8: Mitochondrial ROS is required for NLRP3 inflammasome dependent events

(A) WT BMDM were LPS primed, treated with nigericin following YVAD exposure, and labeled with MitoTracker Red and LysoTracker Green. Cells imaged via live cell microscopy for 1 hr. Data presented as positive cells per field at 60 min. Error bars represent  $\pm$  s.d. n=5 . WT BMDM were LPS primed and treated with nigericin following 100  $\mu$ M YVAD, or 100  $\mu$ M or 500  $\mu$ M MitoTEMPO (Mito T). Supernatants were analyzed for (B) IL-1 $\beta$  via ELISA or (C) LDH release as percentage of LDH release by Triton-X 100 treated control cells. Error bars represent  $\pm$  s.d. n=3 (D) WT BMDM were LPS primed, nigericin and 500  $\mu$ M MitoTEMPO treated. LysoTracker MFI recorded via live cell microscopy. The MFI was normalized to initial signal and background corrected. Data are presented as one line/cell that either underwent MMP (top panel) or retained MitoTracker signal (bottom panel) from five representative fields. (E) WT BMDM were LPS primed and nigericin treated with or without 500  $\mu$ M MitoTEMPO. Cells were labeled with MitoTracker Red and imaged via live cell microscopy. Data are presented as percent of cells/field retaining MitoTracker signal. Error bars represent  $\pm$ s.d. n= 10 fields. (\*\*, P=0.0061).

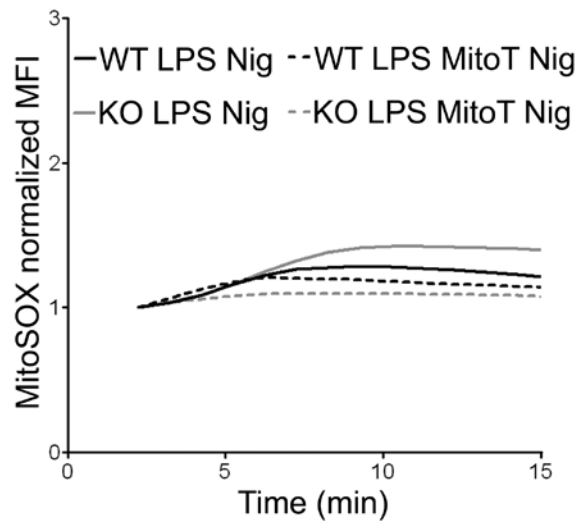


**Figure 2-9:CA-074 Me does not inhibit MMP**

LPS primed and nigericin exposed WT BMDM were either treated with CA-074 Me or left untreated. Cells were labeled with MitoTracker Red and LysoTracker Green. Data were recorded as percent of cells/field that lost signal after one hour of live cell imaging. At least 10 cells per field were counted. Error bars represent  $\pm$  s.d. (n=10 fields for LPS Nig; n=15 fields for LPS Nig CA-074 Me).

### **2.5.7 NLRP3 dependent organelle damage and cytokine release is mediated by mitochondrial ROS**

Next we examined the potential role for mitochondrial ROS in IL-1 $\beta$  processing and secretion, LDH release, LMP, and MMP. To confirm that ROS is generated by mitochondria during inflammasome activation, we used MitoSOX, a dye that specifically detects mitochondrial ROS. Mitochondrial ROS was generated in WT and NLRP3<sup>-/-</sup> BMDM in response to nigericin treatment following LPS priming. MitoTEMPO, a mitochondria specific ROS scavenger, was able to reduce mitochondrial ROS levels slightly (Figure 2-10). 500  $\mu$ M MitoTEMPO reduced both IL-1 $\beta$  secretion and LDH release after nigericin exposure in LPS primed WT BMDM (Figures 2-8B and 2-8C and Table 2-1) and in addition, delayed LMP and reduced subsequent MMP (Figures 2-8D and 2-8E). Together, these data suggest that mitochondrial ROS production is an early NLRP3 inflammasome independent event that may lead to NLRP3 activation, as well as inflammasome dependent LMP, IL-1 $\beta$  secretion, MMP, and cell death.



**Figure 2-10: WT and NLRP3<sup>-/-</sup> BMDM have similar mitochondrial ROS levels**

WT or NLRP3<sup>-/-</sup> BMDM were LPS primed and treated with 20  $\mu$ M nigericin with or without 500  $\mu$ M MitoTEMPO and labeled with MitoSOX. Cells were imaged via live cell microscopy with nigericin added 2 minutes into imaging. Data were normalized to the frame before nigericin addition with background subtraction. ( $n \geq 2$ , ns at 10 min, WT  $\pm$  MitoTEMPO  $P=0.2545$ ; KO  $\pm$  MitoTEMPO  $P=0.0628$ ).



	MITOTRACKER MFI	
	NO LOSS OF MITOTRACKER	LOSS OF MITOTRACKER
<b>LPS NIGERICIN</b>	<b>444.24</b>	<b>123.83</b>
	<b>584.32</b>	<b>124.73</b>
	<b>688.89</b>	<b>117.59</b>
	<b>618.09</b>	<b>103.78</b>
	<b>638.97</b>	<b>141.84</b>
<b>LPS NIGERICIN MITOTEMPO 500 <math>\mu</math>M</b>	<b>1951.8</b>	<b>293.18</b>
	<b>886.22</b>	<b>141.62</b>
	<b>1418.72</b>	<b>117.26</b>
	<b>672.51</b>	<b>156.81</b>
	<b>1315.28</b>	<b>258.45</b>

**Table 2:2: Values of MitoTacker MFI in cells that lost or retained signal**

WT BMDM were LPS primed and treated with nigericin with or without 500  $\mu$ M MitoTEMPO and labeled with MitoTracker Red. MFI with background subtraction was recorded after 60 minutes of live cell wide field imaging. Cells with MFI values <300 were considered to have lost MitoTracker signal as visually verified and used as gating strategy for consistency. Each MFI value recorded is from a single cell from one of five representative fields. (mean MFI in No loss of MitoTracker column = 921.904, s.d.  $\pm$ 482.4133; mean MFI for Loss of MitoTracker column = 157.909, s.d.  $\pm$  64.465).

## 2.6 DISCUSSION

Our results show that in addition to mediating processing and secretion of inflammatory cytokines, NLRP3 inflammasome influences the function and integrity of organelles, cell membrane permeability, and cell death. We examined the kinetics of these events at the single cell level using live cell microscopy in combination with biochemical assays in macrophages exposed to a prototypical NLRP3 activating stimulus. Our data suggest a multi-step process of mitochondrial and lysosomal dysfunction and damage. Nigericin induces rapid mitochondrial depolarization, loss of cellular ATP, and increases in cytosolic ROS levels independently of NLRP3 expression. Depolarization and loss of cellular ATP are unaffected by addition of exogenous KCl, further supporting their NLRP3 independence. Shortly thereafter in treated WT but not NLRP3 deficient cells, lysosomal deacidification is observed and followed kinetically by loss of mitochondrial integrity (MMP) and exposure of PS on the cell surface. ROS derived from mitochondria appear to be responsible at least in part for the observed effects on lysosomes, termed lysosomal membrane permeability (LMP), since the ROS scavenger MitoTempo can delay both LMP and inhibit subsequent MMP. Both LMP and MMP can be inhibited in WT cells by exogenous KCl, which prevents the  $K^+$  efflux required for NLRP3 activation. Based on these results, we propose a model in which some NLRP3 inflammasome activators induce mitochondrial and lysosomal damage, releasing soluble factors from these organelles that sequentially perpetuate inflammasome activation and cell damage. Our data strongly support a role for mitochondrial ROS released into the cytosol as one of these mediators, acting to induce LMP.

Inflammasome-mediated cell death is important for removal of cells infected with intracellular pathogens such as *Salmonella* (Miao et al., 2010). However, the most widely

recognized functions of inflammasomes are to mediate extracellular release of IL-1 cytokine family members. While we and others have been unable to visualize release of these cytokines from individual cells, IL-1 $\beta$  release can be detected in culture supernatants from WT cells by 15 min after nigericin exposure, which closely matches when LMP is first detected in cells. This supports, but does not prove, a role for LMP in IL-1 $\beta$  secretion. Our data do not exclude the possibility that LMP could increase IL-1 $\beta$  release by inducing additional ROS release from mitochondria, and further amplifying the cross-talk pathway with lysosomes (McGuire et al., 2011). We found that when mitochondrial ROS production was inhibited with MitoTEMPO, both LMP and IL-1 $\beta$  secretion were reduced. In contrast, we do not observe MMP and PS exposure in any cells until after IL-1 $\beta$  is readily detectable in culture supernatants, consistent with IL-1 $\beta$  release not requiring cell death.

Since LMP precedes MMP, it could be suggested that released lysosomal products directly damage mitochondria, resulting ultimately in cell death (Boya et al., 2003a; Boya et al., 2003b). The identity of products from lysosomes that could destabilize mitochondria is unclear, but might be suggested to include ROS generated by NADPH oxidases (NOXs) in lysosomes, based on results reported in tumor cells (Kroemer and Jaattela, 2005). However, Nox1-4 deficient macrophages have been shown to produce normal levels of IL-1 $\beta$ , suggesting that lysosomal ROS is not required for NLRP3 inflammasome dependent cytokine release (Dostert et al., 2008; van Bruggen et al., 2010). An additional lysosomal product that might induce mitochondrial dysfunction is cathepsin B (Li et al., 2008), which has been suggested to participate in NLRP3 inflammasome activation after its release from lysosomes into the cytosol in response to particulate inflammasome stimuli (Hornung et al., 2008). Our data place NLRP3 activation upstream of LMP, which is inconsistent with this hypothesis, unless cathepsin B can

be released from intact lysosomes. While able to block IL-1 $\beta$  secretion, the cathepsin B inhibitor CA-074 Me had no effect on MMP. In summary, our data do not support a role for lysosomal products in inducing MMP, but do not exclude this possibility.

An important question raised by our experiments is how mitochondria and their products, including but not limited to ROS, can induce LMP in an NLRP3-dependent fashion. One possibility is suggested by a recent study showing that NLRP3 protein localized in the ER assembles with ASC bound to mitochondria, and that this is mediated through microtubule based movement of mitochondria (Misawa et al., 2013). NLRP3 assembly might thus alter the distribution of mitochondria in the cell, bringing them into proximity with lysosomes, which can also be transported via microtubules (Balint et al., 2013). Lysosomes in WT but not NLRP3 deficient cells might then encounter higher concentrations of mitochondrial ROS or other released products, leading to selective damage and LMP.

In addition to ROS, mitochondrial DNA has been shown to activate the NLRP3 inflammasome (Nakahira et al., 2011). When we labeled mtDNA in BMDM with PicoGreen, we observed loss of signal from mitochondria in WT cells but no signal loss in NLRP3 deficient BMDM. The kinetics were identical to those seen for loss of MitoTracker Red, consistent with loss of mtDNA during MMP. This suggests that mtDNA is released from mitochondria largely after NLRP3 inflammasome is activated, although it does not exclude the possibility that small amounts of mtDNA could be released during initial mitochondrial depolarization.

Our results show that both LMP and MMP in BMDM do not require active caspase-1, since they each are unaffected by the inhibitor YVAD. We have conducted preliminary experiments with BMDM from caspase-1 deficient mice and see partial inhibition of MMP. However, these mice also lack caspase-11, which plays a role in cell death, clouding

interpretation of this data (Aachoui et al., 2013; Case et al., 2013; Shin and Brodsky, 2013). Since YVAD blocks caspase-1 cleavage, uncleaved caspase-1 could potentially mediate cell death, as shown for AIM2 and NLRC4 inflammasomes (Broz et al., 2010). However, NLRP3 lacks the CARD domain needed to directly recruit uncleaved caspase-1.

NLRP3 inflammasome assembles at the ER-mitochondrial interface (Misawa et al., 2013; Zhou et al., 2011) the site where autophagosomes have been recently shown to form in non-immune cells (Hamasaki et al., 2013). Inhibition of autophagy or mitochondrial autophagy (mitophagy) have been shown to increase IL-1 $\beta$  secretion in macrophages, perhaps through the accumulation of damaged mitochondria leading to elevation of cytosolic ROS levels (Naik and Dixit, 2011; Saitoh et al., 2008; Shi et al., 2012). While precisely how IL-1 family cytokines are packaged for secretion remains unclear, we speculate that autophagy might interfere with packaging of IL-1 $\beta$  into specialized secretory vesicles also originating from this site.

### **3.0 ASC IS REQUIRED FOR NLRP3 INFLAMMASOME DEPENDENT PROCESSES AND MEMBRANE DAMAGE**

#### **3.1 ABSTRACT**

Proinflammatory cytokines, such as IL-1 $\beta$ , are produced by innate immune cells, including macrophages and dendritic cells, following detection of pathogenic conditions. These cells utilize multiprotein complexes termed inflammasomes to generate inflammatory cytokines. The NLRP3 inflammasome is the best characterized inflammasome, and is composed of NLRP3, apoptosis-associated speck-like protein with CARD domain (ASC), and caspase-1. ASC acts as an adaptor molecule between the CARD domain of caspase-1 and the pyrin domain of NLRP3, and is required for NLRP3 inflammasome dependent IL-1 $\beta$  processing and secretion. However, it was unknown if ASC was required for sensitivity to membrane damage or membrane repair. Here, we characterized a new system to study ASC, a murine dendritic cell line, D2SC-1, that stably expressed ASC. Using this system, we have shown that ASC is necessary and sufficient to reconstitute the inflammasome in D2SC-1 cells. ASC, but not NLRP3 protein or caspase-1 alone, increased susceptibility to plasma membrane damage during NLRP3 inflammasome activation. Furthermore, ASC increased the susceptibility of cells to lysosomal membrane damage during NLRP3 inflammasome activation, but was not involved with any lysosomal-derived membrane

repair processes. These data demonstrate that ASC plays a crucial role during NLRP3 inflammasome activation.

### 3.2 INTRODUCTION

Inflammation is generated by the release of pro-inflammatory cytokines from innate immune cells such as macrophages and dendritic cells. One of the most important pro-inflammatory cytokines is IL-1 $\beta$ , which is processed and secreted by a multi-protein complex known as the inflammasome. The inflammasome complex includes caspase-1, which is activated by a family of NLR or PYHIN domain containing proteins (Rathinam et al., 2010; Schroder et al., 2010). The best characterized inflammasome is NLRP3, which is triggered by a wide variety of stimuli. NLRP3 contains three domains: leucine-rich repeat, NACHT and pyrin domains (Manji et al., 2002). However, none of these domains permit the binding of NLRP3 to the CARD domain of caspase-1 necessary for caspase-1 oligomerization and activation. Thus, an adaptor that can bridge both components of the inflammasome is required.

Apoptosis-associated speck-like protein with CARD domain (ASC) is an adaptor protein that is essential for NLRP3 inflammasome dependent processing and secretion of IL-1 $\beta$ . ASC is composed of two domains: a pyrin domain and a CARD domain (Martinon et al., 2002). During NLRP3 inflammasome activation, ASC binds to NLRP3 via the pyrin domain and caspase-1 via the CARD domain, thus bridging NLR with caspase-1 (Yu et al., 2006). ASC can act as an adaptor molecule in the formation of other inflammasomes, including AIM2, NLRP2, and NLRP10. The role of ASC in Nlrc4 inflammasome activation is controversial, because Nlrc4 contains a CARD domain, and is thus able to directly bind caspase-1 without ASC. ASC

expression is upregulated by LPS in myeloid cells (Stehlik et al., 2003). Through a mutational study, the CARD domain of ASC was determined to be the minimal unit required for ASC aggregation with caspase-1 in response to both NLRP3 and AIM2 inflammasome stimuli (Proell et al., 2013). It has also been suggested that different splice variants of ASC exist in some cells, and can either promote or inhibit inflammasome formation (Bryan et al., 2010).

As the adaptor bridging NLR with caspase-1, ASC plays a central role in regulating inflammasome activation. While endogenous ASC is normally found in the nucleus of resting cells, it translocates to the cytosol upon exposure to pathogen associated molecular patterns (PAMPs) (Bryan et al., 2009). With extended exposure to bacterial pathogens, cytosolic ASC forms reversible aggregates, or specks, that co-localize with NLRP3 and caspase-1 (Bryan et al., 2009). This speck-like formation is required for the processing and secretion of IL-1 $\beta$ . ASC aggregates were found to mainly occur near the microtubule-organizing center (MTOC) and have been suggested to move along microtubules (Cheng et al., 2010). This has been supported by the recent finding that ASC co-localized with the mitochondria in microtubule dependent NLRP3 inflammasome activation (Misawa et al., 2013). However, it has also been reported that ASC can form a structure known as the “pyroptosome”, which is assembled during cell death without requiring all inflammasome components (Martinon et al., 2009). ASC aggregates are considered distinct from the pyroptosome because ASC aggregation system is reversible and results in the production of IL-1 $\beta$  (Bryan et al., 2009; Martinon et al., 2009).

Although the exact mechanism of NLRP3 inflammasome activation is not fully understood, one possible common stimulus of NLRP3 activation is membrane damage (Philpott and Girardin, 2010). The NLRP3 inflammasome may be able to sense ion efflux, ROS generation, or other events during cell stress resulting from membrane disruption. During



NLRP3 inflammasome activation, several stimuli have been shown to result in membrane damage, including pore-forming toxins and crystals. Furthermore, the ability of cells to repair this damage has been linked to the robustness of the inflammatory response in terms of IL-1 $\beta$  secretion, with cells suffering less membrane disruption resulting in greater IL-1 $\beta$  production (Keyel et al., 2013).

One mechanism that cells might utilize to repair damaged plasma membranes is lysosomal exocytosis. In a study by Huynh et al, it was found that cells used calcium dependent lysosomal exocytosis to repair their membranes from electroporation induced membrane damage (Huynh et al., 2004). This type of membrane repair is dependent on synaptotagmin VII (SytVII) (Reddy et al., 2001). Consistent with these observations, in another study, plasma membrane damage induced by *Mycobacterium tuberculosis*, which also activates the NLRP3 inflammasome, also required calcium dependent movement of lysosomes to the plasma membrane in a SytVII-dependent manner (Divangahi et al., 2009). Similarly, in response to pore-forming toxin induced membrane damage, repair was mediated through Rab-11 mediated exocytosis of vesicles, possibly derived from lysosomes (Los et al., 2011). We have previously shown that lysosomes also sense NLRP3 activation (see Chapter 2), positioning them as potentially key players in NLRP3 inflammasome activation.

In order to characterize the role of ASC during NLRP3 inflammasome activation, we established a model of inflammasome activation using the murine dendritic cell line D2SC-1 (D2). We showed that D2 cells do not contain endogenous ASC, although they do contain the other components of the NLRP3 inflammasome. Stable transduction of ASC in these cells was necessary and sufficient to reconstitute inflammasome activity in these cells. This generated an immortalized cell line that is easier to manipulate than primary murine macrophages or dendritic

cells. We have used this system to show that is ASC required for NLRP3 inflammasome activation following electroporation. Furthermore, ASC increased the susceptibility of cells to both cell and lysosomal membrane damage following NLRP3 activation, suggesting ASC alters membrane repair. However, we did not find any role for ASC in lysosome-mediated membrane repair.

### 3.3 MATERIALS AND METHODS

*BMDM preparation-* Bone marrow was harvested from the tibiae and femora of WT or NLRP3<sup>-/-</sup> B6 mice (gifts from Dr. Lisa Borghesi, Dr. Olivera Finn, and Dr. Timothy Billiar). Bone marrow cells were grown in DMEM (Cellgro) supplemented with 20% fetal bovine serum (FBS) (Gemcell), 2 mM L-glutamine (Cellgro), 500 U penicillin/500 µg streptomycin (Lonza), 1 mM sodium pyruvate (MP Biomedical), and 30% L929 cell (American Type Culture Collection CCL-1) supernatant for 8-25 days. Media was replaced after four days and cells passaged every four days after day 7. For experiments, cells were harvested with Cellstripper (Cellgro) and plated in IMDM (HyClone) supplemented with 10% FBS, 2 mM L-glutamine, 500 U penicillin/500 µg streptomycin (cIMDM). L929 cell supernatants were generated by culturing L929 cells for six days in DMEM supplemented with 10% FBS 2 mM L-glutamine, 500 U penicillin/500 µg streptomycin, 1 mM sodium pyruvate and 1x non-essential amino acids (MP Biomedical). D2SC-1 (D2) cells were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 500 U penicillin/500 µg streptomycin (cDMEM) until confluent. Adherent cells were harvested using Cellstripper (Cellgro). gp293 cells stably expressing *gag* and *pol* from

Moloney murine leukemia virus have been previously described (Burns, J.C et al 1993, PNAS 90(17):8033-8037) and were cultured in cDMEM.

*Generation of stably transfected D2 lines*—ASC was amplified by RT-PCR from murine bone marrow derived macrophages and cloned into the pFB/neo retroviral vector (Stratagene). gp293 cells were co-transfected with VSV-G and either ASC in pFB/neo (D2 ASC) or empty vector (D2 neo) using Lipofectamine<sup>TM</sup> LTX Reagent (Life Technologies), as recommended by the manufacturer with the following modifications: 4 µg of retroviral vector DNA and 2 µg of VSV-G DNA, 5 µl of Plus<sup>TM</sup> reagent, and 10 µl of Lipofectamine<sup>TM</sup> LTX Reagent were mixed with 500 µl of Opti-MEM<sup>®</sup> I Reduced Serum Medium (Life Technologies) per well. After two days, supernatants containing retrovirus were collected and filtered. D2 cells were seeded in a 12-well plate (Costar) at a density of  $2 \times 10^4$  cells/well one day before retrovirus infection. Cells were infected with the recombinant VSV-pseudotyped retrovirus and 24 hours post infection, 1 mg/ml G418 was added to the culture media. After two weeks of selection, G418-resistant cells were expanded and maintained in cDMEM containing 0.5 mg/ml G418.

*TLO Preparation*—Tetanolysin O (Enzo) was reduced by incubation with 10 mM DTT (Sigma). Cells were washed to remove serum prior to the addition of TLO. 100 ng/ml TLO was added in serum free media to appropriate dishes and incubated at 37°C for the duration of imaging as indicated.

*LDH Release Assay*—D2 neo or D2 ASC were plated one day before the experiment at 500,000 cells/well in a 12 well tissue culture plate in cIMDM. Cells were incubated for 4 hours with 1 µg/ml LPS *E. coli* 026:B6 (Sigma L2654). 50 mM KCl, 100 µM zVAD or 100 µM Ac-YVAD-CMK (Alexis) were added during the last 30 minutes of LPS treatment and then re-added

when media was replaced. Cells were treated with 20  $\mu$ M nigericin sodium salt (Sigma) in 1 ml phenol red-free cDMEM and incubated at 37°C for 5-30 minutes. 1% Triton X-100 (Fisher Scientific) was added to cells for five minutes as a positive control for complete cell lysis. Supernatants were removed and assayed in duplicate using an LDH cytotoxicity kit (Cayman Chemical) for OD using a PowerWave XS microplate spectrophotometer (BioTek). Percent LDH release was calculated as (mean OD value of sample-mean OD value of blank)/(mean OD value of TritonX-100 control sample – mean OD of blank) x 100.

*Electroporation*—D2 neo, D2 ASC, wild-type (WT) BMDM, or NLRP3<sup>-/-</sup> BMDM were plated at 450,000 cells/ well in a six-well non-tissue culture treated plate one night before the experiment and incubated at 37°C. Media was removed from wells and DMEM was added to the wells. Cells were then harvested using Cellstripper, centrifuged, and resuspended in 450  $\mu$ l electroporation buffer (2% FBS, 10 mM Hepes, pH 7 in Hank's balanced salt solution). Cells were placed on ice until transferred to electroporation cuvettes (BTX Harvard Apparatus 2mm space) and electroporated on a BTX Electroporation System at 200 V with a capacitance of 600  $\mu$ F, and resistance of 246. Cells were then incubated at 37°C for 1 min or 30 min before being placed back on ice.

*Flow cytometry*—D2 neo, D2 ASC, WT BMDM, or NLRP3<sup>-/-</sup> BMDM were plated and LPS primed as described above. Cells were treated with 20  $\mu$ M nigericin, TLO, 3 mM ATP (Fisher), 50 mM KCl, and/or 100  $\mu$ M of the cathepsin B inhibitor CA-074 Me (Calbiochem). In some experiments, cells were also electroporated as described above. Cellstripper was used to harvest cells, which were then centrifuged at 1200 rpm for 10 min at 4°C.

For the live/dead assay, supernatants were discarded and pellets were resuspended in 1x PBS. Calcein and ethidium bromide were added to samples and incubated at 4 C for 1 hr.

Samples were then analyzed on an BD LSRII flow cytometer using the FITC and PE-A channels with compensation. 10,000 events were collected/sample. FlowJo (Treestar) was used for data analysis.

For Lamp-1 surface expression, cells were stained with mouse anti-Lamp-1 conjugated to Alexa Fluor 488 (Life Technologies) at a 1:100 dilution for 30 minutes on ice, washed, fixed in 2% paraformaldehyde and analyzed using the FITC channel on an LSR II.

*Immunofluorescence*—Cells were harvested one day before the experiment and plated on autoclaved 12 mm round coverslips (Fisher) coated with Poly L-Lysine (Sigma) in a 12 well plate at 500,000 cells/well and incubated overnight at 37°C. Media was replaced with DMEM. Cells were primed for 4 hrs with 1 µg/ml LPS and then treated with either 20 µM nigericin or TLO for 15-30 minutes at 37°C. Cells were fixed on ice with 4% paraformaldehyde (Electron Microscopy Sciences). Cells were permeabilized with 0.1% Triton in 1x PBS, washed, blocked in 2% BSA and then stained for 1 hr with anti-ASC mouse antibody clone 2E1-7 (Millipore) at 1:200 dilution in 100 µl final volume/coverslip on ice. Cells were next washed five times in 0.5% BSA, stained for 1 hr with secondary anti-mouse Alexa Fluor 647 antibody (Life Technologies) at 1:1000 dilution on ice. Cells were washed three times in 0.5% BSA and then counterstained with Dapi (Sigma 5 mg/ml stock) at 1:5000 for 30 seconds, washed two more times and mounted in gelvatol (gift from the Center for Biologic Imaging) on Superfrost® Plus Microscope slide (Fisher) and stored at 4°C. Slides were imaged on an Olympus Fluoview 1000 confocal microscope and images analyzed with MetaMorph software.

*Live cell microscopy*—D2 ASC or D2 neo cells were plated at 250,000 cells/dish in cDMEM in 35 mm collagen coated glass bottom culture dishes (MatTek) one night before the experiment. Cells were primed with 1 µg/ml LPS for three to four hours in fresh media.

Inhibitors were added during the last 30 minutes of LPS priming at 37°C and were added back to the dish in fresh media before imaging, after washing out LPS-containing media. 2 μM LysoTracker Green DND-26 (Life Technologies) was added during the last 30 minutes of LPS priming. Dishes were imaged on Nikon A1 inverted microscope with a Plan Fluor 40x objective with an 1.30 NA or Plan Apo 60x objective with an 1.40 NA, Photometrics Coolsnap HQ2 or Andor™ Technology iXon<sup>EM</sup>+ camera, NikonPiezo driven XYZ stage, and Tokai Hit Environmental chamber. Images were captured every 30 seconds for one hour. 20 μM nigericin or TLO were added 2 frames into imaging in 1ml of media. Videos were analyzed using Elements (Nikon) and Excel (Microsoft).

*IL-1β ELISA*—D2 neo or D2 ASC cells were plated at 500,000 cells/well in a 12 well tissue culture plate one night before the experiment. Cells were LPS primed for four hours and treated as described above. Supernatants were harvested and IL-1β levels assayed by ELISA. ELISA plates were read at 450 nm with 570 nm background subtraction using BioTek® Powerwave XS Microplate Spectrophotometer and Gen5™ Data Analysis Software.

*SDS-PAGE and Western Blot*—Lysates and supernatants were collected from  $2.0 \times 10^6$  BMDM, D2, D2 neo, or D2 ASC cells after treating cells as described above in serum-free media. Lysates were prepared using NP-40 lysis buffer (Boston BioProducts) containing a protease inhibitor cocktail (Calbiochem). Supernatants were TCA precipitated and both lysates and supernatants were analyzed for IL-β via Western blot as previously described (Chu JLB 2009). Briefly, the entire precipitated supernatant or 50 μg of protein of lysate was loaded onto an 11% polyacrylamide gel. Samples were run using a Labnet Power Station 300 and then transferred onto a PVDF membrane (Thermo) using the iBlot (Invitrogen) system according to the manufacturer's instructions. Western blotting was performed using the SNAPid system

(Millipore) according to the manufacturer's directions. Mouse antibodies against NLRP3 (Enzo), ASC (Millipore clone 2E1-7), or IL-1 $\beta$  (3ZD clone from National Cancer Institute) were used as primary antibodies and goat antibodies against mouse conjugated to HRP (Santa Cruz) was used as a secondary antibody at a 1:1666 dilution. Enhanced chemiluminescence reagent (Santa Cruz) was added before imaging on a Kodak Image Station 4000MM (Molecular Imaging Systems).

*Statistical analysis*—All data sets were analyzed via a student's two-tailed t-test unless otherwise noted using Prism software (Graphpad). P values were as indicated and were considered significant if  $P < 0.05$ .

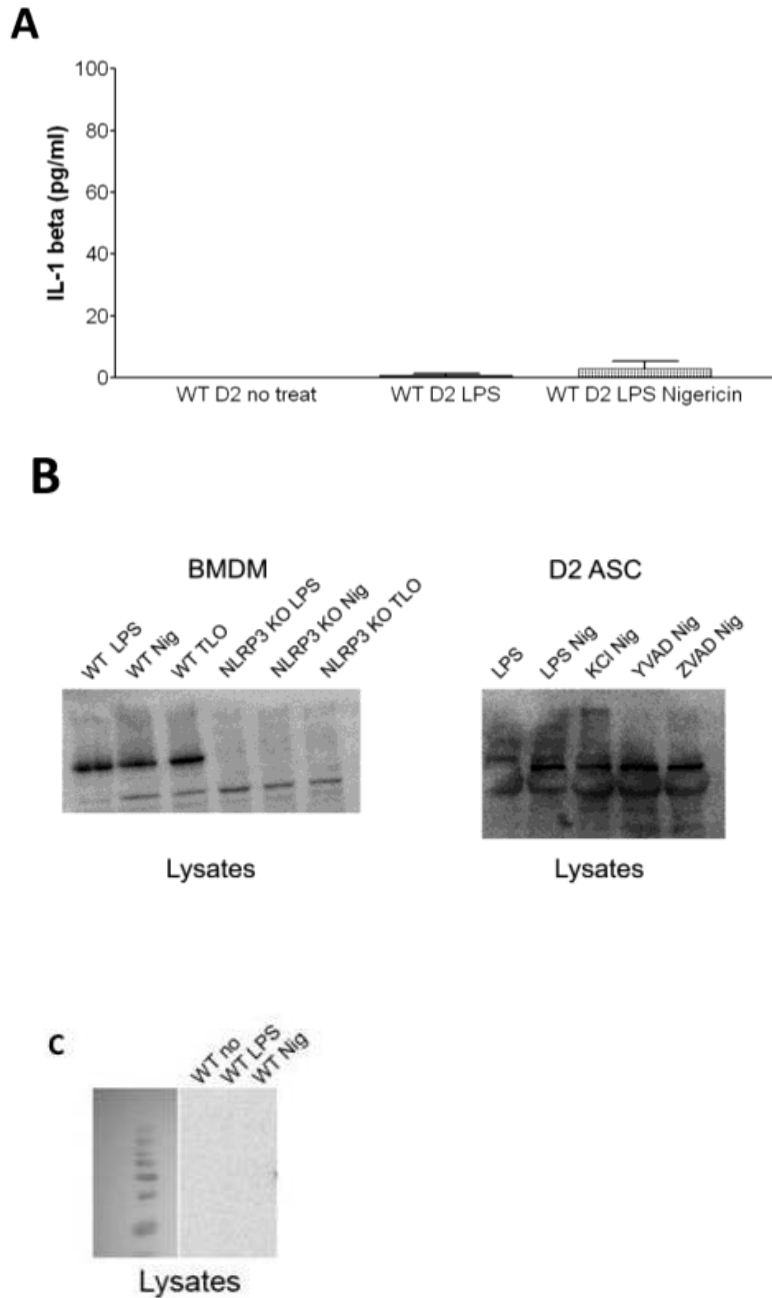
## 3.4 RESULTS

### 3.4.1 Wild-type D2 cells are unable to process and secrete IL-1 $\beta$

Previously, we had characterized NLRP3 activation in bone marrow derived macrophages (BMDM) (Heid et al., 2013). In order to characterize the NLRP3 inflammasome in a dendritic cell system we used the immortalized cell line D2-SC1 (D2). This cell line also enabled us to avoid the limitation of using primary cells. In addition to macrophages, the NLRP3 inflammasome is commonly found in dendritic cells, from which D2 cells are derived. Since these cells were derived from immune cells, we first characterized the ability of these cells to produce mature IL-1 $\beta$ . The potassium ionophore nigericin is a prototypical activator of the NLRP3 inflammasome and produces robust secretion of mature IL-1 $\beta$  from wild-type (WT) LPS BMDM (see Chapter 2). In contrast to this robust secretion of mature IL-1 $\beta$  from WT BMDM, LPS primed WT D2 cells did not secrete significant levels of IL-1 $\beta$  following exposure to

nigericin (Figure 3-1A). A second stimulus of the NLRP3 inflammasome, the pore-forming toxin tetanolysin O (TLO), also failed to induced IL-1 $\beta$  secretion in LPS primed WT D2 cells (data not shown). These data indicate that WT D2 cells, although derived from inflammasome-competent dendritic cells, lack a functional inflammasome.





**Figure 3-1: WT D2 do not have a functional NLRP3 inflammasome**

(A) WT D2 were LPS primed or unprimed and stimulated with 20  $\mu$ M nigericin for 30 min. IL-1 $\beta$  in supernatants was measured via ELISA. n=2 (B) WT BMDM, NLRP3<sup>-/-</sup> BMDM (left panel), or D2 ASC (right panel), were LPS primed for four hours and then treated with 20  $\mu$ M nigericin or TLO with or without the inhibitors KCl, YVAD and zVAD. Cells were lysed and 50 $\mu$ g lysate was resolved by SDS-PAGE prior to transfer to PVDF. NLRP3 protein

was detected via western blot at about 117 kD. (C) WT D2 were LPS primed or unprimed and then treated with 20  $\mu$ M nigericin for 30 min. Cells were lysed and 50 $\mu$ g lysate was resolved by SDS-PAGE prior to transfer to PVDF. ASC protein levels was detected via Western blot. Size ladder shown on far left.

### **3.4.2 WT D2 cells do not express ASC**

Since D2 cells did not appear to have a functional inflammasome based on lack of IL-1 $\beta$  secretion, we determined whether D2 cells have the components of the NLRP3 inflammasome. We measured NLRP3 protein expression in D2 cells via western blot analysis. NLRP3 was detected in LPS primed D2 cells (Figure 3-1B). The addition of extracellular potassium chloride, and the inhibition of caspase-1 activity via the caspase 1 inhibitor YVAD, or the pan-caspase inhibitor ZVAD, are known to significantly ablate NLRP3 inflammasome dependent IL-1 $\beta$  processing and secretion in bone marrow derived macrophages (BMDM). However, treatment of D2 cells with these inhibitors prior to nigericin stimulation did not diminish NLRP3 proteins levels in D2. Since the NLRP3 inflammasome is well-characterized in BMDM, and genetic knockouts exist, we measured protein levels of NLRP3 in these cells. NLRP3 protein expression was detected in WT BMDM, but not in NLRP3<sup>-/-</sup> BMDM, indicating that the antibody was specific for NLRP3.

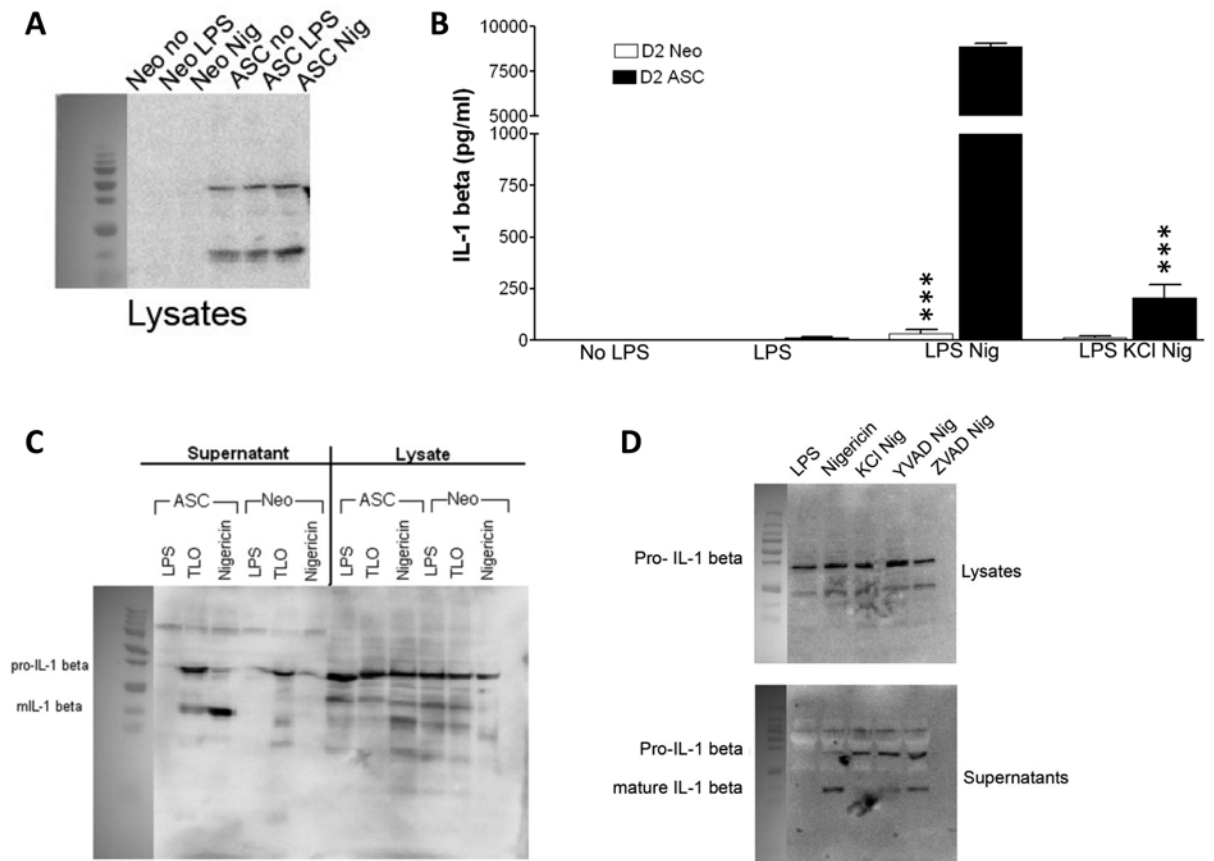
To explore what other components of the NLRP3 inflammasome D2 cells possess, we examined the level of ASC protein via western blot analysis. ASC is the adaptor molecule that binds to NLRP3 via the pyrin domain and caspase-1 via CARD domain, and is necessary for NLRP3 inflammasome function. ASC was not present in D2 cells (Figure 3-1C). Therefore, although D2 cells possess the NLRP3 protein, they may be unable to form an inflammasome complex due to the absence of ASC.

### 3.4.3 Transduced D2 ASC cells have a functional NLRP3 inflammasome

To test the hypothesis that ASC was needed to reconstitute the NLRP3 inflammasome in D2 cells, we transduced D2 cells with ASC. Western blot analysis verified that ASC protein was present in transduced D2 cells, but not in a cell line transduced with the empty vector, D2 Neo (Figure 3-2A). ASC protein was detected in D2 ASC cells that were not LPS primed, suggesting it is constitutively expressed in D2 cells (Figure 3-2A). In contrast, WT BMDM upregulate ASC expression upon LPS priming. To verify that D2 ASC cells had a functional NLRP3 inflammasome, we examined IL-1 $\beta$  processing and secretion in LPS primed, nigericin treated cells. Significant levels of IL-1 $\beta$  were detected in D2 ASC cells following nigericin treatment, but not in D2 Neo control cells (Figure 3-2B). Furthermore, nigericin stimulation alone did not trigger IL-1 $\beta$  secretion in D2 ASC cells, consistent with the need for LPS priming to induce IL-1 $\beta$  protein expression. High levels of extracellular KCl also blocked the secretion of IL-1 $\beta$  in LPS primed nigericin treated D2 ASC, suggesting that IL-1 $\beta$  secretion was dependent on the NLRP3 inflammasome.

In addition to IL-1 $\beta$  secretion, NLRP3 inflammasome activation is known to result in the processing of pro-IL- $\beta$  to mature IL-1 $\beta$  via caspase-1 before it is secreted from the cell. In order to confirm that the IL-1 $\beta$  secreted from D2 ASC cells was processed, we performed western blot analysis of D2 ASC supernatants. Pro-IL-1 $\beta$  was detected in the lysates of D2 neo and D2 ASC cells (Figure 3-2C). When LPS primed and treated with nigericin, only D2 ASC cells processed and secreted IL-1 $\beta$  (Figure 3-2C). Moreover, preventing potassium efflux from the cell or inhibiting caspase-1 activity decreased mature IL-1 $\beta$  in the supernatants of D2 ASC cells. This suggested that NLRP3 inflammasome activation is regulated by similar control mechanisms as observed for WT BMDM (Figure 3-2D). TLO induced mature IL-1 $\beta$  in the supernatants of D2

ASC, but not D2 neo. There was also a corresponding decrease of pro-IL-1 $\beta$  in the lysates of those samples. Taken together, these data demonstrate that ASC is sufficient to reconstitute the NLRP3 inflammasome in D2 cells.



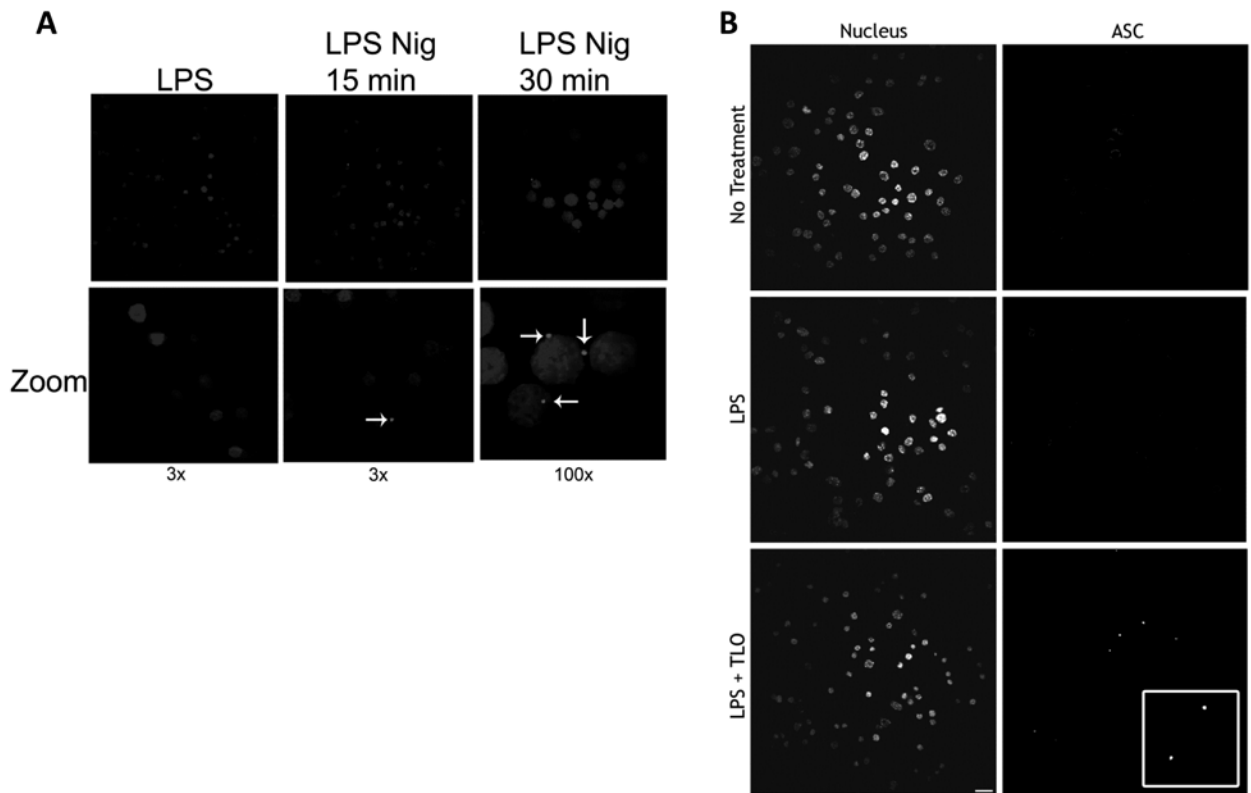
**Figure 3-2: Transduction of D2 cells with ASC reconstitutes functional NLRP3 inflammasome**

(A) D2 neo or D2 ASC cells were LPS primed or left unprimed and treated with 20  $\mu$ M nigericin for 30 min. Cells were lysed and 50 $\mu$ g lysate was resolved by SDS-PAGE prior to transfer to PVDF. ASC protein levels were detected via western blot (bottom band). Size ladder shown on far left. (B) D2 neo or D2 ASC cells were LPS primed or left unprimed and then treated with 20  $\mu$ M nigericin for 30 min with or without 50 mM KCl. IL-1 $\beta$  was detected via ELISA. (n=3; LPS Nig D2 neo vs D2 ASC, \*\*\*, P<0.001; D2 ASC LPS KCl Nig vs D2 ASC, \*\*\* P<0.0001) (C, D) D2 ASC or D2 neo (panel C only) cells were LPS primed and treated with nigericin or TLO with

inhibitors where indicated. Cells were lysed and 50µg lysate or entire TCA-precipitated supernatant was resolved by SDS-PAGE prior to transfer to PVDF. IL-1β protein levels were detected via Western blot. Size ladder shown on far left.

#### **3.4.4 NLRP3 inflammasome induction results in ASC aggregation**

Since ASC was necessary and sufficient to reconstitute NLRP3 inflammasome activation in D2 cells, we next asked where ASC was localized during inflammasome activation. Previously, it was reported that ASC moves from the nucleus to the cytoplasm to form speck-like aggregations in response to NLRP3 inflammasome activation. However, it is not known whether these specks form before or after inflammasome activation and IL-1β processing. We examined ASC via confocal microscopy, and found that it was not expressed in D2 neo cells, as expected. D2 ASC cells showed extremely faint, diffuse, cytosolic ASC staining when untreated or LPS primed only. Within 15 minutes of nigericin stimulation of LPS-primed D2 ASC cells, ASC formed punctuate structures in the cell, usually with only one speck per cell (Figure 3-3A). The number of cells with speck-like structures of ASC increased by 30 minutes of nigericin treatment. Speck-like structures of ASC were also observed in TLO-treated, LPS-primed D2 ASC cells (Figure 3-3B). These results demonstrate that NLRP3 inflammasome activation promotes ASC aggregation into specks that could be functional inflammasomes.



**Figure 3-3:D2 ASC cells show ASC aggregation following NLRP3 inflammasome activation**

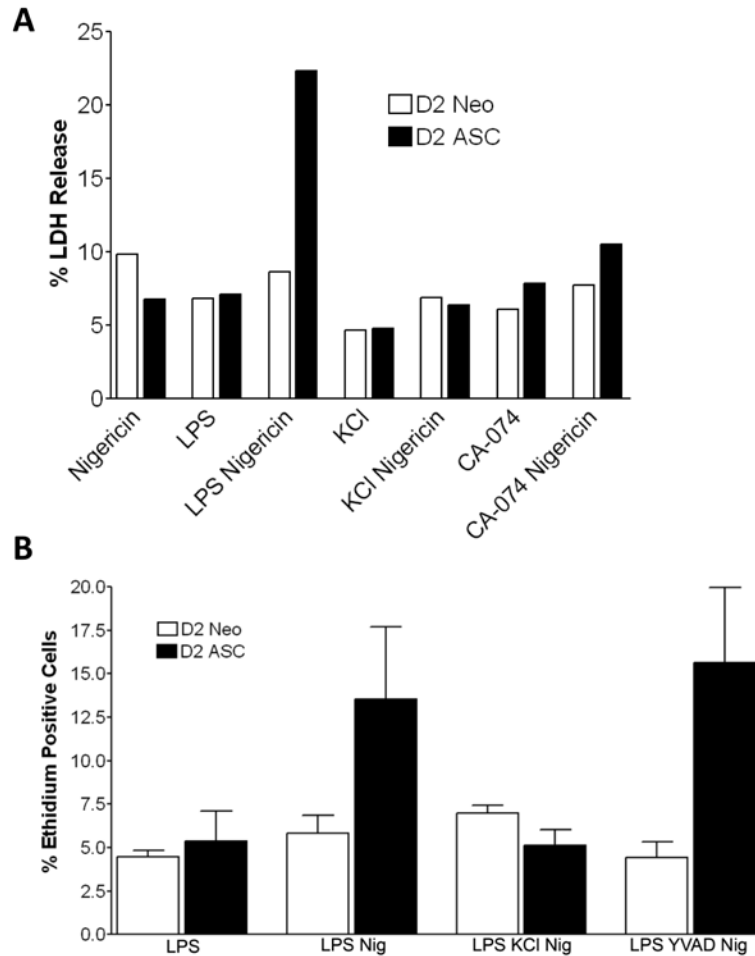
(A) LPS primed D2 were treated with nigericin for 15 or 30 minutes, fixed and stained for ASC and nuclei (DAPI). Cells were imaged confocal microscopy. Bottom panels show higher magnification. Arrows point to ASC aggregates. (B) D2 ASC were LPS primed or unprimed, stimulated with a sublytic dose of TLO for 30 min, fixed and stained with DAPI (left panels) and anti-ASC antibody (right panels). Inset shows higher magnification. Scale bar = 20  $\mu$ m.

### 3.4.5 Inflammasome induced membrane permeability is ASC and NLRP3 dependent

In addition to evaluation of cytokine secretion and speck formation, D2 ASC cells provide a useful model system for examining membrane damage. LDH release from BMDM indicates membrane damage and occurs in a NLRP3 inflammasome dependent process (see Chapter 2). To test if this occurred in D2 ASC cells, we measured LDH release into the supernatants following nigericin induced NLRP3 inflammasome activation of LPS-primed cells. There were higher levels of LDH released into the supernatant of D2 ASC cells than D2 neo cells (Figure 3-4A). This release was decreased through the inhibition of potassium efflux, lack of LPS priming, or the addition of CA-074 Me, a cathepsin B inhibitor known to block NLRP3 inflammasome dependent IL-1 $\beta$  processing and secretion (Hornung et al., 2008). Significantly less LDH was released into the supernatants of D2 neo cells, further confirming that this process required the NLRP3 inflammasome.

To further explore if ASC was responsible for the increased sensitivity to membrane damage during NLRP3 inflammasome activation, we used a cell viability assay to measure membrane permeability and cell death. In response to nigericin induced NLRP3 activation, D2 ASC cells had a greater amount of membrane permeability than D2 neo control cells (Figure 3-4B). Membrane permeability in D2 ASC could be significantly decreased with the inhibition of potassium efflux, further confirming that this was an NLRP3 inflammasome-dependent event. Increased membrane damage in D2 ASC occurred within five minutes of exposure to the NLRP3 inflammasome stimulus (Figure 3-5A). Similarly, WT BMDM had a greater extent of membrane damage than NLRP3<sup>-/-</sup> BMDM (Figure 3-5B). However, inhibition of caspase-1 via the YVAD or the use of caspase-1<sup>-/-</sup> BMDM did not show a comparable reduction in membrane damage, suggesting that caspase-1 alone is not involved with NLRP3-dependent membrane

damage. These data demonstrated that both NLRP3 and ASC are responsible for increased susceptibility to membrane damage following NLRP3 inflammasome activation.



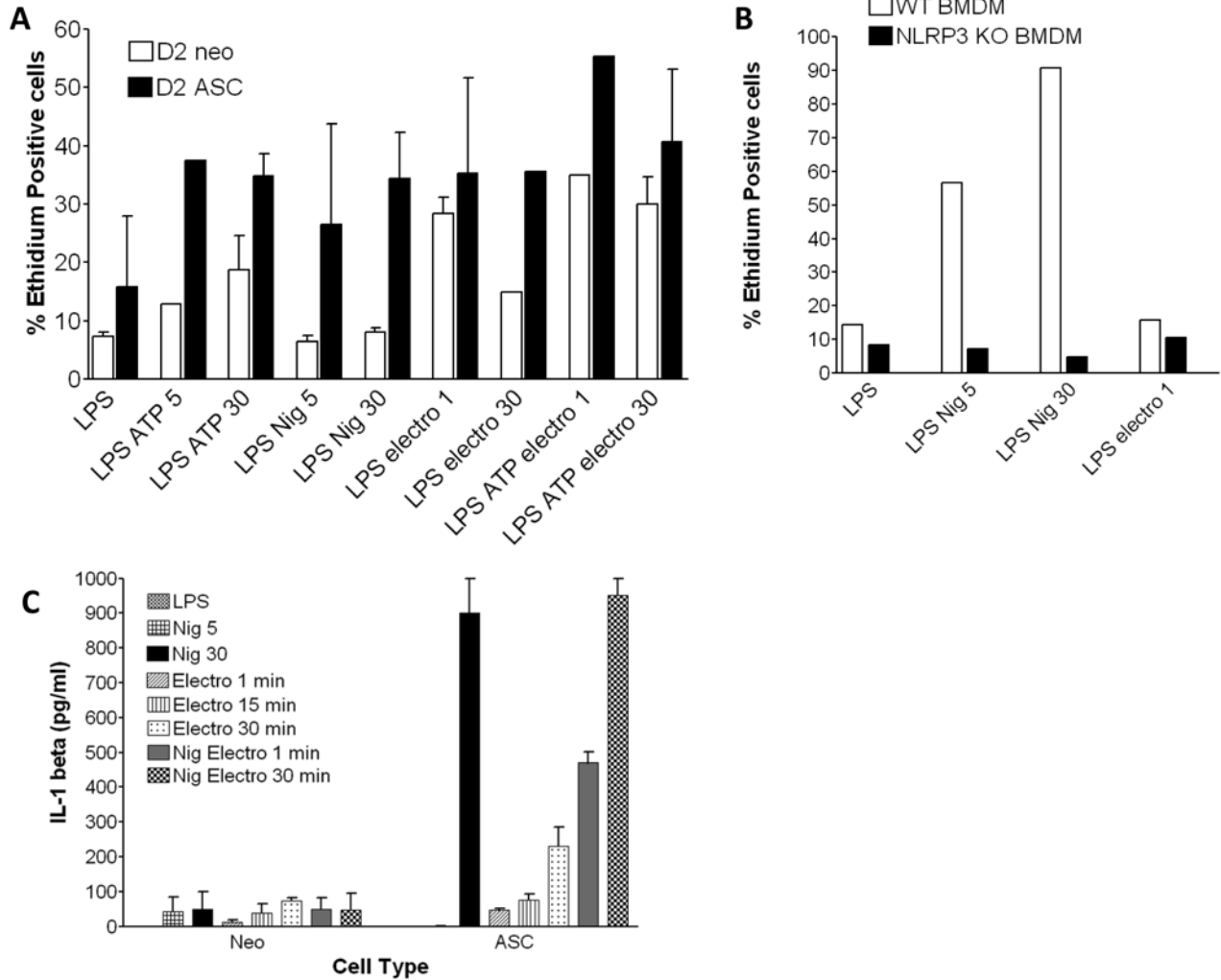
**Figure 3-4:ASC increases D2 cell sensitivity to membrane damage following NLRP3 inflammasome activation**

(A) D2 neo or D2 ASC cells were LPS primed or left unprimed and then treated with nigericin for 30 minutes with or without inhibitors. LDH release was measured from cell supernatants. Data are represented as percent of LDH released from Triton treated control cells. Data from one representative assay shown, each sample was an average of two wells. (B) D2 ASC or D2 neo cells were LPS primed, treated with nigericin for 30 minutes with or without



inhibitors and Live/dead assay was performed using flow cytometry. Data are represented as percentage of ethidium positive cells. n=2

To determine whether the observed sensitivity to membrane damage was due entirely to the NLRP3 agonists, or due to general membrane damage, we examined the ability of LPS-primed D2 neo or D2 ASC cells to survive electroporation. We found that D2 neo were less susceptible to membrane damage 30 minutes following electroporation than D2 ASC, despite equivalent membrane damage in D2 neo and D2 ASC at only one minute following electroporation (Figure 3-5A). However, both WT and NLRP3<sup>-/-</sup> BMDM appeared more resistant than D2 cells at one minute following electroporation, suggesting an intrinsic difference in cell type (Figure 3-5B). D2 ASC secreted IL-1 $\beta$  in response to electroporation, while D2 neo did not, demonstrating that electroporation activated the inflammasome (Figure 3-5C). ATP is a known activator of the NLRP3 inflammasome. When LPS-primed D2 ASC cells were first treated with ATP, and then electroporated, they showed increased levels of membrane damage over similarly treated D2 neo cells (Figure 3-5A). These data suggest that membrane damage itself can induce NLRP3 inflammasome activation, and that once activated, ASC can increase the susceptibility of those cells to membrane permeability.

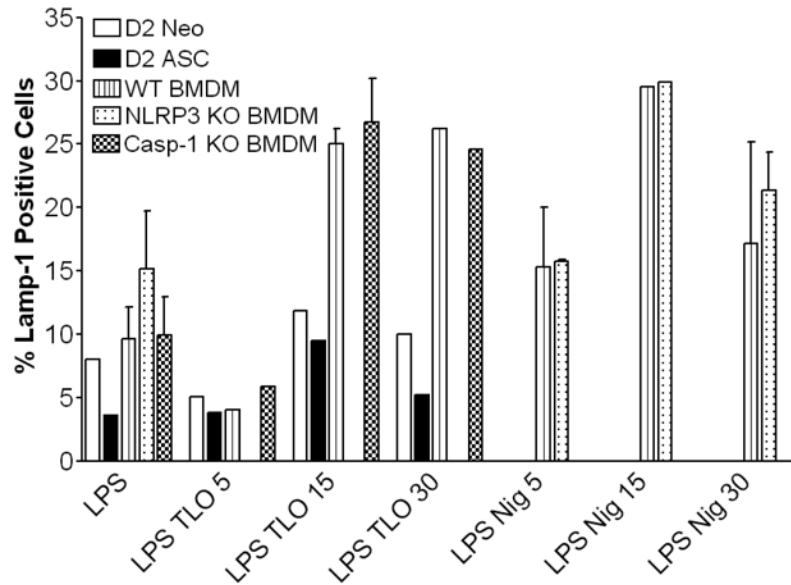


**Figure 3-5: Presence of active NLRP3 inflammasome increases membrane damage to various stimuli**

(A) D2 neo or D2 ASC cells were LPS primed and either treated with 20  $\mu$ m nigericin or 3mM ATP for five or 30 minutes, or electroporated followed by one or 30 minutes of recovery at 37°C. The percentage of ethidium positive cells was measured by live/dead cell assay. (B) WT or NLRP3<sup>-/-</sup> BMDM were LPS primed and treated with nigericin for five or 30 minutes or electroporated and then incubated at 37°C for 1 min. The percentage of ethidium positive cells was measured by live/dead cell assay. Data from one representative experiment are shown. (C) D2 neo or D2 ASC cells were LPS primed and treated with nigericin for 5 or 30 minutes, or electroporated and incubated at 37°C for 1, 15, or 30 min. Some cells were treated with nigericin first for 30 minutes and then electroporated, followed by recovery at 37°C for 1 or 30 min. IL-1 $\beta$  in supernatants was measured via ELISA. n=2

### **3.4.6 ASC increases susceptibility of cells to lysosomal damage during NLRP3 inflammasome activation**

It had previously been suggested that lysosomal exocytosis could be used as a mechanism of membrane repair in damaged cells (Reddy et al., 2001). Since the increase in membrane permeability could be due to either an increase in membrane damage or a decrease in membrane repair, we looked for evidence of lysosome-mediated membrane repair in D2 neo and D2 ASC cells. However, there was no significant difference in the amount of the lysosomal membrane protein Lamp-1 on the surface of LPS-primed, TLO-treated D2 ASC and D2 neo cells (Figure 3-6). Furthermore, surface levels of Lamp-1 were no different between D2 ASC and D2 neo cells that were LPS primed and electroporated (data not shown). Therefore, our data did not suggest that lysosomes were being used to repair the cell in D2 ASC cells during NLRP3 inflammasome activation. Lamp-1 surface levels did not increase under conditions of membrane damage in WT, NLRP3<sup>-/-</sup>, or caspase-1<sup>-/-</sup> BMDM (Figure 3-6). These data are consistent with reports showing that endocytic structures other than lysosomes are capable of mediating membrane repair (Cerny et al., 2004).

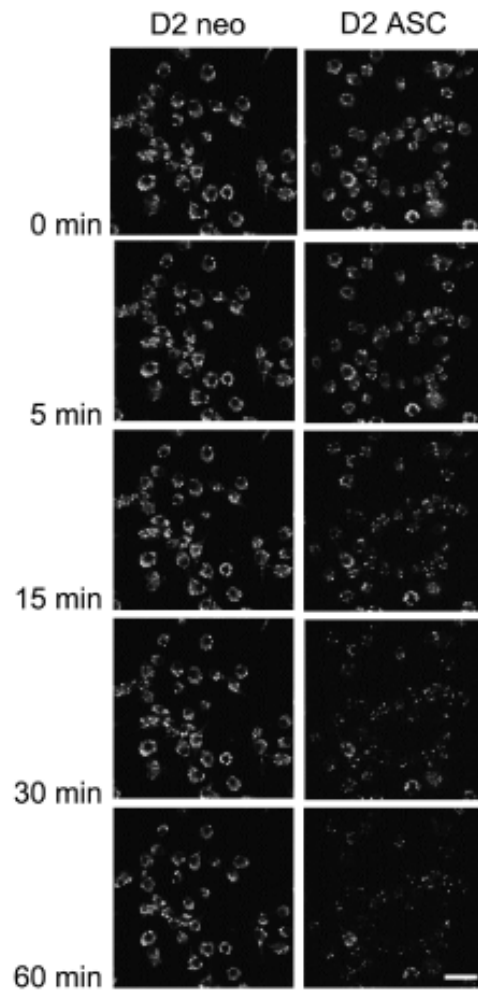


**Figure 3-6: No evidence for Lamp-1 mediated cell membrane repair following NLRP3 inflammasome activation**

D2 neo, D2 ASC, WT BMDM, Caspase-1<sup>-/-</sup> BMDM, or NLRP3<sup>-/-</sup> BMDM were LPS primed and treated with TLO or nigericin for 5, 15, or 30 minutes. Lamp-1 surface expression was measured via flow cytometry. The percentage of Lamp-1 positive cells is shown.

Although lysosomes may not play a role in membrane repair in D2 cells, lysosomes are also the main source of cathepsin B. Since our cathepsin B inhibitor significantly reduced NLRP3 inflammasome induced LDH release in D2 ASC cells, we asked what happened to

lysosomes during inflammasome activation. We labeled lysosomes in LPS primed D2 neo and D2 ASC with LysoTracker green and then imaged them via live cell microscopy. We found that lysosomes in D2 ASC treated with TLO, but not in D2 neo, lost LysoTracker signal (Figure 3-7). This is indicative of lysosomal membrane permeability (LMP). Therefore, our results show that although there is no evidence for lysosome-mediated repair in D2 ASC cells, there is ASC-dependent destabilization of the lysosomal membrane in response to NLRP3 inflammasome activation.



**Figure 3-7: ASC required for NLRP3 inflammasome dependent LMP**

D2 neo or D2 ASC cells were LPS primed and then treated with a sublytic dose of TLO. Lysosomes were labeled with LysoTracker Green. Images were taken at 0, 5, 15, 30 and 60 minutes post-TLO treatment. One representative field out of five is shown. Scale bar= 50  $\mu$ m

### 3.5 DISCUSSION

We have shown ASC is necessary and sufficient to reconstitute the NLRP3 inflammasome in D2SC-1 cells. D2 ASC cells process and secrete IL-1 $\beta$  in response to TLO and nigericin following LPS priming. We have used this system to demonstrate that electroporation results in the activation of the NLRP3 inflammasome and secretion of IL-1 $\beta$ . Furthermore, we found that D2 ASC cells have an increased susceptibility to membrane damage following NLRP3 inflammasome activation. Although both plasma membrane damage and lysosomal membrane permeability (LMP) occurred, we found no evidence that membrane repair requires lysosomal exocytosis in these cells. This is a novel system for studying the role of ASC in the NLRP3 inflammasome.

The D2 system will allow the dissection of the molecular mechanism of inflammasome activation. This system recapitulates the inflammasome, as shown by the expression of inflammasome proteins and tests of function. One inhibitor of the NLRP3 inflammasome is KCl, which blocks potassium efflux from the cell. Potassium efflux from the cell is a common requirement for NLRP3 inflammasome activation using many different stimuli (Kanneganti and Lamkanfi, 2013). Although the mechanism is not fully understood, potassium efflux may promote the physical assembly of the NLRP3 inflammasome complex. The data presented here support this idea. When we block potassium efflux from the cell using KCl, we observe that NLRP3 dependent IL-1 $\beta$  processing and secretion, LDH release, and cell membrane damage are abrogated. We have shown that KCl does not alter levels of NLRP3 or pro-IL-1 $\beta$  present in cell lysates, suggesting that its inhibitory effect occurs at a post-translational level. This could occur through preventing the NLRP3 inflammasome complex from assembling. These results

recapitulate what has been observed in BMDM, and allow for future mechanistic studies of inflammasome function using this cell system.

One finding with this system was that membrane damage induced by electroporation was sufficient to induce secretion of IL-1 $\beta$ . Since this did not occur in D2 neo cells, we believe this to be an inflammasome dependent event. This membrane damage could have generated a sufficient efflux of potassium from the cell to promote the assembly of NLRP3 inflammasome components. In contrast, caspase-1 inhibition, which was able to block the processing and secretion of IL-1 $\beta$ , did not prevent NLRP3 inflammasome dependent cell membrane damage. These data suggest that caspase-1 activity does not play a role in NLRP3 inflammasome dependent membrane susceptibility, but its proteolytic ability is required for IL-1 $\beta$  processing. These findings are consistent with those shown with the AIM2 inflammasome indicating that the catalytic domain of caspase-1 was not necessary for pyroptosis (Broz et al., 2010).

This model system shows that the NLRP3 inflammasome activation may reduce the ability of cells to repair membrane damage. After only one minute of recovery following electroporation, both D2 neo and D2 ASC cells had similar levels of permeabilized cells, as shown by positive ethidium staining. However, after 30 minutes of recovery, there were fewer permeabilized cells in the D2 neo population, but not in the D2 ASC population. Although it is possible this difference could be due to the loss of dead cells from the assay, both D2 ASC and D2 neo cells retain similar morphology, making this possibility unlikely. More likely, D2 neo are better able to repair their membranes better than D2 ASC. This is likely due to the activation of the NLRP3 inflammasome in D2 ASC, but not D2 neo. The D2 ASC execute an inflammatory response that includes pyroptosis, shown by IL-1 $\beta$  release, rather than the repair mechanisms engaged by the D2 neo. Activation of the NLRP3 inflammasome prior to electroporation further



increased membrane damage in D2 ASC cells. This further supports the idea that membrane repair mechanisms in the cell are diverted to an inflammatory response through previous NLRP3 inflammasome activation.

Lysosomes are implicated in membrane repair (Reddy et al., 2001). However, we found that Lamp-1 did not accumulate at surface, suggesting lysosomes played no role in membrane repair in the D2 system. Instead, it is possible that lysosomes contribute to membrane damage via LMP. Lysosomal membrane permeability following inflammasome activation is dependent on NLRP3 (see chap 2). The data here extend these findings to a second system and verify that ASC is required for LMP in addition to NLRP3. This suggests that LMP requires the functional NLRP3 inflammasome, instead of simply NLRP3 protein. Interestingly, our D2 ASC cells lost LysoTracker signal slightly later than WT BMDM, indicating that kinetics of these NLRP3 inflammasome events varies by cell type. In both cell types, it is evident that LMP occurs downstream of NLRP3 inflammasome activation and is dependent on both NLRP3 and ASC present in the cell. This LMP could result in the release of lysosomal products, such as cathepsin B into the cytoplasm. The cathepsin B inhibitor, CA-074 Me, was able to significantly decrease LDH release in D2 ASC cells treated with nigericin. These results, combined with the absence of Lamp-1 surface expression observed following inflammasome activation, suggest that lysosomes and their contents could contribute to cell damage during NLRP3 inflammasome activation rather than lysosomal mediated repair. In conclusion, we have generated a novel cell system that can be used to elucidate inflammasome function.

### **3.6 ACKNOWLEDGEMENTS**

I would like to thank Dr. Chengqun Sun (Department of Immunology, University of Pittsburgh) for the generation of the D2 ASC cell line, all the transductions and constructs required for this study, instruction on cell culture and molecular biology, as well as invaluable discussion. I would also like to thank former Salter lab members, Dr. Jessica Chu, Dr. L. Mike Thomas, and Dr. Peter Keyel (Department of Immunology, University of Pittsburgh) for their helpful discussion and advice for data analysis. Finally, I would like to thank my mentor, Dr. Russell Salter (Department of Immunology, University of Pittsburgh) for his guidance, ideas, discussion, and suggestions throughout the entirety of this study.

## **4.0 ATP INDUCED NLRP3 INFLAMMASOME DEPENDENT IL-1 BETA SECRETION AND ORGANELLE DAMAGE REQUIRES MITOCHONDRIAL ROS AND P2X7**

### **4.1 AUTHOR CONTRIBUTIONS**

The majority of the work in this study was published in the *Journal of Immunology* (*published ahead of print October 2, 2013, doi: 10.4049/jimmunol.1301490; Copyright 2013. The American Association of Immunologists, Inc.*). Michelle E. Heid (Department of Immunology, University of Pittsburgh) wrote the manuscript, designed experiments, conducted the majority of the experiments, and analyzed most of the data. Peter A. Keyel (Department of Immunology, University of Pittsburgh) assisted with live cell microscopy analysis. Christelle Kamga and Sruti Shiva (Department of Pharmacology and Chemical Biology, University of Pittsburgh) provided useful discussion of mitochondrial metabolism. Simon C. Watkins (Department of Cell Biology, University of Pittsburgh) assisted with live cell microscopy and provided technical advice and helpful discussion throughout the project. Russell D. Salter (Department of Immunology, University of Pittsburgh) was the Principal Investigator for the project, provided mentorship, advice, and assistance with writing throughout the entire project. I would also like to acknowledge Lisa Borghesi (Department of Immunology, University of Pittsburgh) for providing WT bone marrow, Tim Billiar (Department of Surgery, University of Pittsburgh) for providing

NLRP3<sup>-/-</sup> bone marrow and George Dubyak (Department of Physiology and Biophysics, Case Western Reserve University) for providing P2X7<sup>-/-</sup> bone marrow.

## 4.2 ABSTRACT

Extracellular ATP induces a potent inflammatory response when released from damaged tissue. ATP mediates this inflammation through activation of the purinergic receptor, P2X7. P2X7 engagement activates the NLRP3 inflammasome, which leads to the processing and secretion of the pro-inflammatory cytokine IL-1 $\beta$  in macrophages. The pathway and kinetics of this activation remain unknown. Here, we have shown using live cell imaging that ATP stimulation of LPS primed bone marrow derived macrophages triggers an intracellular cascade involving organelle cross-talk prior to the processing and secretion of IL-1 $\beta$ . ATP triggered NLRP3 independent mitochondrial depolarization, followed by NLRP3 dependent lysosomal membrane permeability (LMP), and mitochondrial membrane permeability (MMP). Mitochondrial ROS was required for continued ATP dependent lysosomal damage, MMP, and IL-1 $\beta$  secretion. These NLRP3 dependent responses to ATP required the P2X7 receptor, but not the closely related P2X4 receptor. In contrast, the potassium ionophore nigericin required P2X4, but not P2X7, for NLRP3 activation and IL-1 $\beta$  secretion. These results demonstrate that ATP induced inflammasome activation requires mitochondrial ROS. This study extends mechanistic insights into the NLRP3 inflammasome activation, demonstrating how ATP is distinct from other NLRP3 inflammasome agonists in terms of purinergic receptor requirements.

### 4.3 INTRODUCTION

Sterile inflammation is inflammation produced without the presence of pathogen-associated molecular patterns and plays a major role in many diseases, including atherosclerosis and autoimmunity. Sterile inflammation can be driven by a number of altered endogenous molecules, such as oxidized LDL, or factors that are only released following cellular damage, such as ATP. ATP is a particularly important inflammatory mediator that can be released following cell or tissue damage. ATP mediates its inflammatory properties through engagement of purinergic receptors, especially the P2X7 receptor (Riteau et al., 2012).

Engagement of the P2X7 receptor via ATP creates a non-selective pore in the cell membrane that allows ions, such as calcium and potassium, to pass through (Rayah et al., 2012). The P2X7 pore has been proposed to involve pannexin, although pannexin may be dispensable for pore formation (Browne et al., 2013; Sun et al., 2013). Importantly, pannexin is not always required for mature IL-1 $\beta$  secretion in response to ATP or nigericin in murine bone marrow derived macrophages (BMDM) (Pelegriin et al., 2008; Qu et al., 2011). In addition to ion transport, engagement of P2X7 via ATP has been reported to increase cellular ROS (Cruz et al., 2007) and to activate the NLRP3 inflammasome (Riteau et al., 2012).

The NLRP3 inflammasome is a multiprotein complex consisting of the sensing protein NLRP3, adaptor ASC and effector caspase-1, which promotes cell death and the processing of pro-inflammatory cytokines. In addition to ATP, the NLRP3 inflammasome is activated by a variety of diverse stimuli, including nigericin, pore forming toxins, and crystals, among other

substances (Bauernfeind et al., 2011a). The diverse nature of these stimuli does not lend clarity to the nature of NLRP3 inflammasome activation. There is inconclusive evidence that activators of the inflammasome directly bind NLRP3. More likely, these stimuli create a common cellular stress response through diverse mechanisms. One common requirement for all known inflammasome stimuli is the efflux of potassium from the cell (Munoz-Planillo et al., 2013; Perregaux and Gabel, 1994). Adding 50 mM KCl to most cell culture conditions will inhibit NLRP3 inflammasome dependent IL- $\beta$  processing and secretion. The opening of the P2X7 pore could explain how ATP treatment mediates potassium efflux during NLRP3 inflammasome activation (Kahlenberg and Dubyak, 2004). In addition to potassium efflux, the production of reactive oxygen species (ROS) has been considered a common requirement for many of the NLRP3 stimuli (Davis and Ting, 2010). Although ROS production is a common feature of most NLRP3 stimuli, the ability of P2X7 to directly generate ROS may provide a unique impetus in ATP treated cells that could directly activate the NLRP3 inflammasome. ATP stimulation of the NLRP3 inflammasome is completely dependent on the purinergic receptor P2X7. P2X7<sup>-/-</sup> BMDM do not secrete mature IL-1 $\beta$  in response to ATP, but do in response to other stimuli, such as nigericin. Therefore, ATP stimulation of the NLRP3 inflammasome via P2X7 activation can utilize a unique method of NLRP3 inflammasome activation.

Early studies using ATP stimulation to activate the NLRP3 inflammasome found that the high concentrations of ATP required for P2X7 activation (3 mM) led to optimal IL-1 $\beta$  processing and secretion over lower concentrations of ATP (1 mM), which activate the P2X4 receptor (Qu et al., 2007; Raouf et al., 2007). Furthermore, macrophages treated with lower ATP concentrations secreted more cathepsin B than cells treated with higher concentrations. These data suggest that reduced amounts of ATP exposure in LPS-primed cells lead to more lysosomal

leakiness, possibly due to P2X4, rather than NLRP3 inflammasome activation. P2X4 has been reported to be located on the lysosomes in microglial cells (Qureshi et al., 2007). Therefore, treatment of cells with ATP may directly induce lysosomal damage, in addition to NLRP3 inflammasome activation, depending on the concentration of ATP used. The physiologically relevant concentration of ATP remains controversial. The existence of ATP concentration gradients around cells treated with 3 mM ATP could result in some cells stimulated at 3mM via P2X7, while other cells further away could be activated at lower ATP concentrations via P2X4. In this study, we characterized the response of BMDM to 3 mM ATP treatment. We found that ATP led to NLRP3 independent mitochondrial depolarization, followed by NLRP3 dependent mitochondrial dysfunction and permeability. Furthermore, ATP also induced lysosomal damage. This organelle damage and IL-1 $\beta$  secretion required mitochondrial ROS generation in response to ATP treatment. These results are consistent with those observed for other inflammasome stimuli, such as nigericin. Organelle damage and IL-1 $\beta$  secretion induced by ATP required P2X7, but not P2X4. Surprisingly, inhibition of P2X4 blocked nigericin-induced, NLRP3-dependent IL-1 $\beta$  secretion. This finding suggests that lower concentrations of ATP leaking from damaged cells may play a more general role in NLRP3 inflammasome activation.

#### 4.4 MATERIALS AND METHODS

*BMDM preparation-* Bone marrow was harvested from the tibiae and femora of WT, NLRP3<sup>-/-</sup> or P2X7<sup>-/-</sup> B6 mice (gifts from Dr. Lisa Borghesi, Dr. Olivera Finn, and Dr. Timothy Billiar). Bone marrow cells were grown in DMEM (Cellgro) supplemented with 20% fetal bovine serum (FBS) (Gemcell), 2 mM L-glutamine (Cellgro), 500 U penicillin/500  $\mu$ g streptomycin (Lonza), 1

mM sodium pyruvate (MP Biomedical), and 30% L929 cell (American Type Culture Collection CCL-1) supernatant for 8-25 days. Media was replaced after four days and cells passaged every four days after day 7. For experiments, cells were harvested with Cellstripper (Cellgro) and plated in IMDM (HyClone) supplemented with 10% FBS, 2 mM L-glutamine, 500 U penicillin/500 µg streptomycin (cIMDM). L929 cell supernatants were generated by culturing L929 cells for six days in DMEM supplemented with 10% FBS 2 mM L-glutamine, 500 U penicillin/500 µg streptomycin, 1 mM sodium pyruvate and 1x non-essential amino acids (MP Biomedical).

*MTT assay*- WT BMDM were plated one day before the experiment at 500,000 cells/well in a 12 well tissue culture plate (Costar) in cIMDM, as described above. Cells were incubated for 4 hours with 1 µg/ml LPS from *E. coli* 026:B6 (Sigma L2654). 50 mM KCl, 100 µM or 500 µM MitoTEMPO (Enzo) were added during the last 30 minutes of LPS treatment and then re-added when media was removed and 1 ml fresh phenol red-free cIMDM (Gibco) was added to the cells. 20 µM nigericin sodium salt (Sigma) or 3 mM ATP (Sigma) was added to the cells and incubated at 37°C for 5-30 minutes. After supernatants were removed, cells were washed once with 1x DPBS (Cellgro) and analyzed using the Vybrant® MTT cell proliferation assay kit (Life Technologies) according to the manufacturer's instructions and then assayed in duplicate for OD using a PowerWave XS microplate spectrophotometer (BioTek). The percentage of MTT unconverted to formazan in the sample was calculated as  $100 - (\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{blank}}) / (\text{mean OD}_{\text{untreated control}} - \text{mean OD}_{\text{blank}}) \times 100$ .

*LDH Release Assay*- WT BMDM were treated as described above for the MTT assay. 1% Triton X-100 (Fisher Scientific) was incubated with cells for five minutes as a positive control for complete cell lysis. Supernatants were removed and assayed in duplicate using an LDH



cytotoxicity kit (Cayman Chemical) for OD using a PowerWave XS microplate spectrophotometer. Percent LDH release was calculated as  $(\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{blank}}) / (\text{mean OD}_{\text{positive control}} - \text{mean OD}_{\text{blank}}) \times 100$ .

*Live cell microscopy*- WT BMDM were plated at 250,000 cells/dish in 35 mm collagen coated glass bottom culture dishes (MatTek) one night before the experiment in cIMDM. Cells were LPS-primed (1  $\mu\text{g/ml}$ ) for three to four hours in fresh media. Inhibitors were added during the last 30 minutes of LPS priming at 37°C and were added back to the dish in fresh media before imaging after washing out LPS-containing media. 2 $\mu\text{M}$  MitoTracker Red CM-H2XRos (LifeTechnologies) or 2  $\mu\text{M}$  LysoTracker Green DND-26 (LifeTechnologies) were added during the last 30 minutes of LPS priming. 6.5  $\mu\text{M}$  JC-1 was added during the last 20 minutes of LPS priming. Dishes were imaged on Nikon TiE or Nikon A1 inverted microscope with an 1.30 NA Plan Fluor 40x objective or an 1.40 NA Plan Apo 60x objective, Photometrics Coolsnap HQ2 or Andor™ Technology iXon<sup>EM</sup> + camera, Nikon Piezo driven XYZ stage, and Tokai Hit Environmental chamber. Images were captured every 30 seconds for one hour. 20  $\mu\text{M}$  nigericin or 3 mM ATP was added 2 minutes into imaging in 1 ml of media. Videos were analyzed using Elements (Nikon) and Excel (Microsoft).

*IL-1 $\beta$  ELISA*- WT, NLRP3<sup>-/-</sup>, or P2X7<sup>-/-</sup> BMDM were plated at 500,000 cells/well in a 12 well tissue culture plate one night before. Cells were LPS-primed for four hours and treated as indicated. 100 $\mu\text{M}$  A740003 (Tocris) or 50 or 100 $\mu\text{M}$  5-BDBD (Tocris) were added as indicated. Supernatants were analyzed by IL-1 $\beta$  ELISA as follows. A 96 well plate was coated in 4  $\mu\text{g/ml}$  of purified mouse anti-IL-1 $\beta$  capture antibody (eBioscience) at 4°C overnight. The wells were blocked in 1% filtered BSA (Sigma) for two hours at room temperature and then samples were added to the wells for two hours at room temperature. Biotin conjugated anti-IL-1 $\beta$  mouse

detection antibody (eBioscience) was added at 6  $\mu\text{g/ml}$  for one hour at room temperature. Avidin-HRP (Biolegend) was added to wells at 1:5000 for 30 minutes at room temperature. Equal volumes of TMB (Biolegend) reagent A and B were mixed and then 100  $\mu\text{l}$  was added to each well for no more than 20 minutes at room temperature. 50  $\mu\text{l}$  of 1M sulfuric acid was added to wells to stop the reaction. OD values were read at 450 nm with 570 nm background subtraction using BioTek® PowerWave XS Microplate Spectrophotometer and Gen5™ Data Analysis Software.

*Statistical analysis-* All data sets were analyzed via a student's two-tailed t-test unless otherwise noted using Prism (Graphpad). Indicated P values were considered significant if  $P < 0.05$ . Significance confirmed with ANOVA.

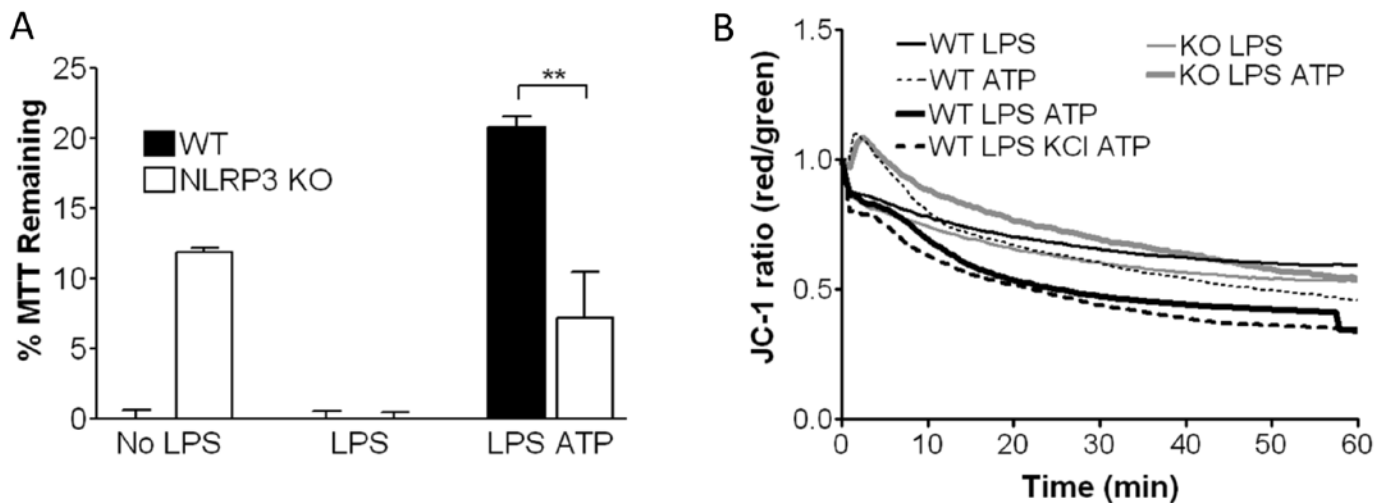
## 4.5 RESULTS

### 4.5.1 Mitochondrial dysfunction results from ATP stimulation in LPS primed cells

Since NLRP3 activation by many stimuli is linked to mitochondrial dysfunction, we tested whether ATP similarly induced mitochondrial dysfunction. We primed WT and NLRP3<sup>-/-</sup> BMDM with LPS to upregulate NLRP3 inflammasome components and then treated them with 3 mM ATP, a dose known to induce NLRP3 inflammasome dependent IL-1 $\beta$  processing and secretion (Qu et al., 2007). Mitochondrial function was assessed by measuring the amount of MTT converted to formazan via mitochondrial reductases. LPS priming alone did not affect the ability of either WT or NLRP3<sup>-/-</sup> BMDM to convert MTT to formazan (Figure 4-1A). However, following LPS priming and ATP treatment, only NLRP3<sup>-/-</sup> BMDM, but not WT BMDM, had

functional mitochondrial reductases able to convert MTT to formazan (Figure 4-1A). This result suggests that ATP activation of the NLRP3 inflammasome reduced the function of mitochondrial reductases.

To further explore mitochondrial function during NLRP3 inflammasome activation, we evaluated mitochondrial depolarization using the live cell dye JC-1. This ratiometric dye fluoresces red when aggregated in polarized mitochondria and green when in nonpolarized mitochondria in a monomeric form. We found that both WT and NLRP3<sup>-/-</sup> BMDM showed mitochondrial depolarization in response to LPS-priming and ATP (Figure 4-1B). Depolarization was enhanced in cells exposed to ATP alone, although LPS priming alone also produced some depolarization (Figure 4-1B). ATP induced depolarization could not be significantly diminished through the inhibition of potassium efflux with potassium chloride (KCl) (Figure 4-1B). Together, these results indicate that ATP induces mitochondrial depolarization independently of NLRP3, but increases mitochondrial dysfunction in cells with an active NLRP3 inflammasome in terms of mitochondrial reductase activity.



**Figure 4-1: ATP induces both NLRP3 dependent and independent mitochondrial dysfunction**

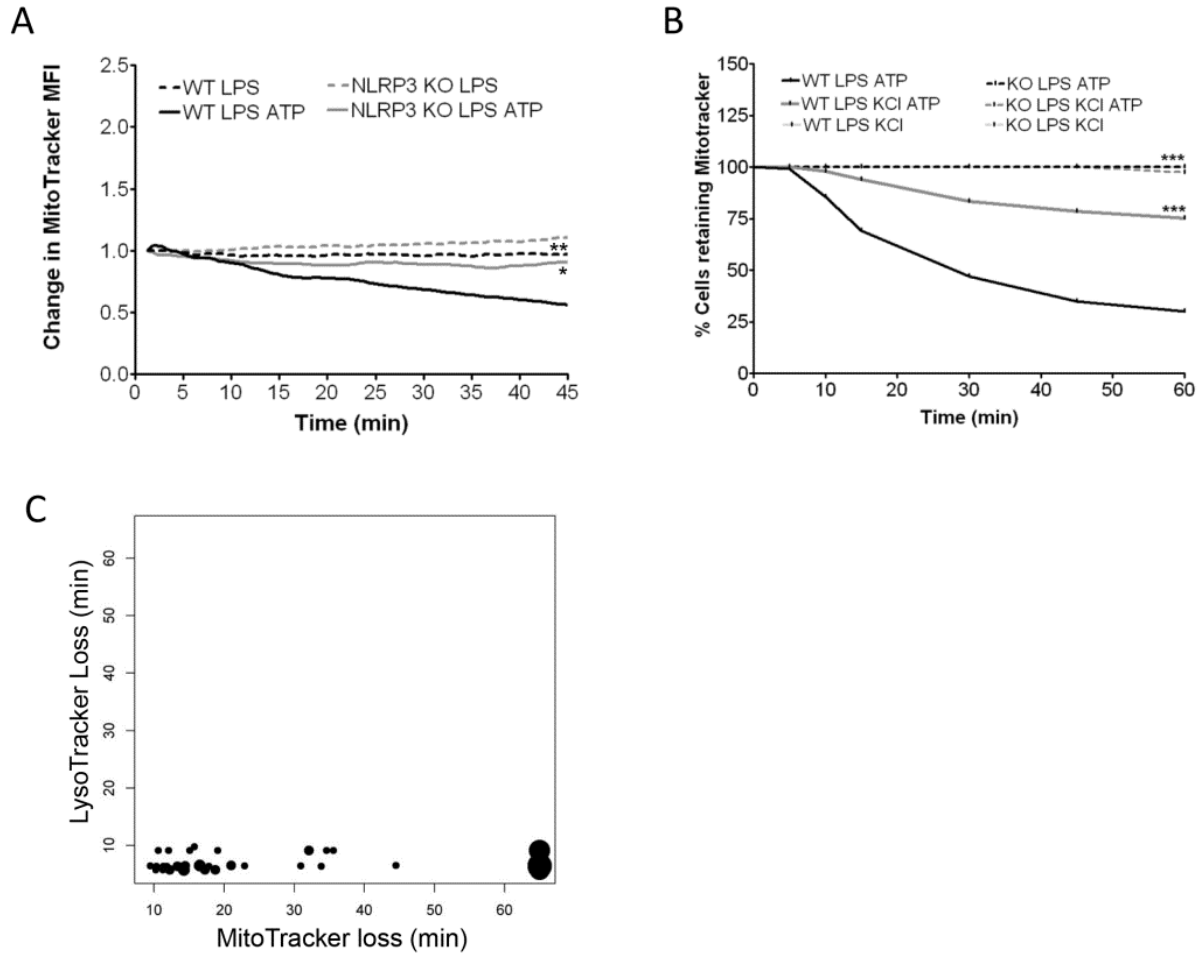
(A) LPS-primed or unprimed WT and NLRP3<sup>-/-</sup> BMDM were treated with 3 mM ATP for thirty minutes and assayed for their ability to convert MTT to formazan and graphed as percentage of MTT reagent remaining unconverted by mitochondrial reductases. Readings were taken as an average of two wells. Error bars represent mean  $\pm$  s.d. (n=3, \*\*, P=0.0155). (B) LPS-primed or unprimed WT and NLRP3<sup>-/-</sup> BMDM were treated with 3 mM ATP with or without 50 mM KCl for one hour. Mitochondrial depolarization was measured by calculating the ratio of red to green JC-1 signal during wide field live cell imaging. (n=5)

#### **4.5.2 ATP induced loss of mitochondrial membrane integrity is NLRP3 dependent**

Since ATP-induced both NLRP3 dependent and independent mitochondrial dysfunction, we further explored the effects of ATP on the mitochondrion. Mitochondrial membrane permeability (MMP) can be measured via the retention of mitochondrial specific dyes, such as MitoTracker red. When we treated LPS primed WT BMDM with ATP and examined mitochondrial integrity by live cell microscopy, we found a decrease in MitoTracker red signal over time (Figure 4-2A). This MMP was not seen in similarly treated NLRP3<sup>-/-</sup> BMDM or in WT BMDM treated with LPS alone. We further quantitated these data by measuring the percentage of cells in each field that retained mitochondrial membrane integrity following one hour of ATP exposure. Following LPS priming and ATP treatment, a higher percentage of cells retained MitoTracker signal in NLRP3<sup>-/-</sup> BMDM than in WT BMDM (Figure 4-2B). The inhibition of potassium efflux from the cells completely prevented MMP in LPS primed and ATP treated WT BMDM (Figure 4-2B). Together, these results indicate that ATP-induced MMP is a NLRP3 dependent event, suggesting that NLRP3 activation leads to severe mitochondrial damage.

### **4.5.3 LMP precedes MMP in response to ATP induced inflammasome activation**

Closely related to mitochondrial permeability and dysfunction during NLRP3 activation is lysosomal membrane permeability (LMP) (see Chapter 2). We tested whether LMP occurred following ATP induced inflammasome activation by live cell imaging LPS-primed and ATP treated WT or NLRP3<sup>-/-</sup> BMDM stained with the lysosomal marker LysoTracker Green. In WT BMDM, we observed the loss of LysoTracker Green (Figure 4-2C). This loss was NLRP3 dependent, because LMP did not occur in similarly treated NLRP3<sup>-/-</sup> BMDM (data not shown). When LPS primed WT BMDM were labeled with both LysoTracker Green and MitoTracker Red, LMP was found to always precede MMP, with LMP occurring within the first ten minutes of ATP treatment. MMP occurred around 15-30 minutes after ATP exposure for most cells. Furthermore, while all cells analyzed lost lysosomal membrane integrity, only a subset of cells underwent MMP (Figure 4-2C). These data suggest that both lysosomal and mitochondrial membrane damage are NLRP3 dependent events induced by ATP stimulation of the inflammasome.



**Figure 4-2: LMP and MMP are ATP induced NLRP3 inflammasome dependent events**

(A) LPS-primed WT or NLRP3<sup>-/-</sup> BMDM were left untreated or treated with 3 mM ATP and stained with MitoTracker Red. Cells were imaged for 45 minutes after ATP addition via live cell microscopy. The loss of MitoTracker red signal over time was recorded and data were normalized to the initial signal following background subtraction. (n≥9, at 45 min \*, P=0.0382 for WT LPS ATP vs NLRP3<sup>-/-</sup> LPS ATP; \*\*, P=0.0021 for WT LPS vs WT LPS ATP). (B) WT or NLRP3<sup>-/-</sup> (KO) BMDM were LPS-primed, treated with 3 mM ATP with or without 50 mM KCl and stained with MitoTracker Red. Cells were imaged for one hour after ATP addition via live cell microscopy. The percentage of cells/field retaining MitoTracker Red signal after 0, 5, 10, 15, 30, 45, and 60 min are displayed. All lines not easily seen are at 100% behind the dashed line. (n≥5, \*\*\*, P< 0.0001 for WT LPS ATP vs WT LPS KCl ATP and KO LPS ATP vs WT LPS ATP). (C) WT BMDM were LPS-primed, treated with 3 mM ATP and stained with MitoTracker Red and LysoTracker Green. Cells were imaged for one hour after ATP addition via live cell microscopy. Data were recorded as time to loss of signal, where dot size corresponds to the number of cells

losing signal at any given time point. Cells that did not lose MitoTracker Red were recorded as data points beyond the one hour imaging period. Data from five fields are displayed.

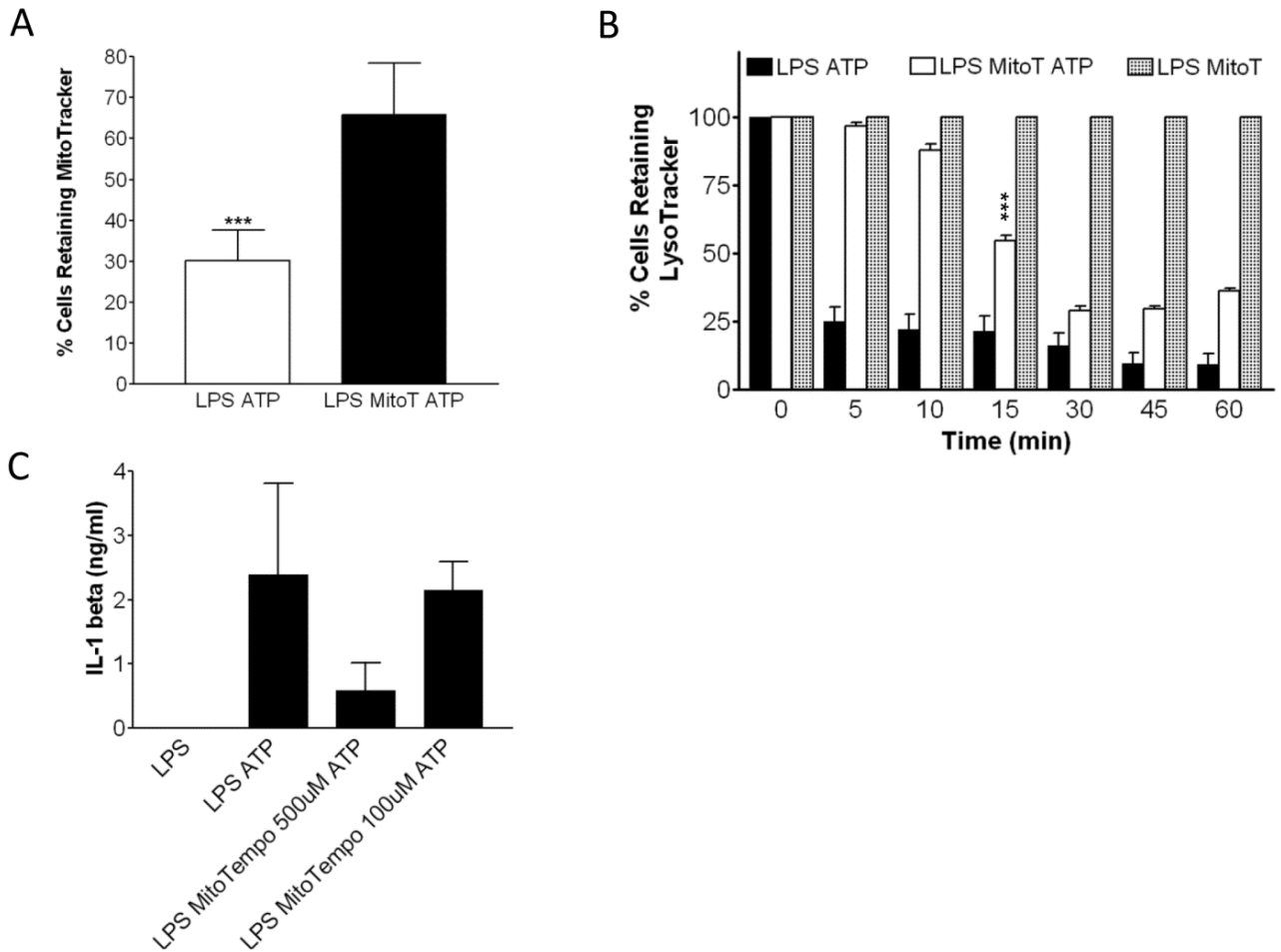
#### **4.5.4 Loss of mitochondrial and lysosomal membrane integrity requires mitochondrial ROS**

We have observed NLRP3 independent mitochondrial depolarization and NLRP3 dependent LMP followed by NLRP3 dependent MMP. Mitochondrial depolarization releases reactive oxygen species (ROS). Mitochondrial ROS can activate the NLRP3 inflammasome (Zhou et al., 2011), suggesting that mitochondrial ROS could mediate the NLRP3-dependent effects of ATP. We tested this hypothesis using LPS primed WT BMDM that were treated with ATP in the presence of the specific mitochondrial ROS inhibitor MitoTEMPO (Nakahira et al., 2011). In the presence of 500  $\mu$ M MitoTEMPO, ATP induced MMP was significantly reduced (Figure 4-3A). This suggests that mitochondrial ROS played a role in damage to the mitochondrial membrane.

Since LMP is an NLRP3 dependent event induced by ATP that precedes MMP, we asked whether mitochondrial ROS impaired LMP. We found that LMP was reduced in LPS primed WT BMDM treated with ATP in the presence of MitoTEMPO (Figure 4-3B). Furthermore, what LMP did occur in the presence of MitoTEMPO was delayed until after 15 minutes, rather than occurring within five minutes of ATP treatment as observed in control cells. There was also a slight increase in the percentage of LysoTracker positive cells that were treated with MitoTEMPO from 45 minutes to 60 minutes after ATP exposure (Figure 4-3B). These data indicate that LMP is controlled by mitochondrial ROS.

Since mitochondrial ROS were required for both LMP and MMP during NLRP3 activation, we next asked if mitochondrial ROS were necessary for ATP induced IL-1 $\beta$

processing and secretion. The processing and secretion of IL-1 $\beta$  is a hallmark of NLRP3 inflammasome activation. We measured IL-1 $\beta$  secretion in LPS primed and ATP treated WT BMDM with and without MitoTEMPO. We found that treatment with 500  $\mu$ M MitoTEMPO significantly inhibited the secretion of IL-1 $\beta$  (Figure 4-3C). Together, these data suggest ATP-dependent IL-1 $\beta$  secretion, LMP, and MMP require mitochondrial ROS following NLRP3 activation by ATP.



**Figure 4-3: Mitochondrial ROS is required during NLRP3 dependent organelle damage and IL-1 $\beta$  secretion**



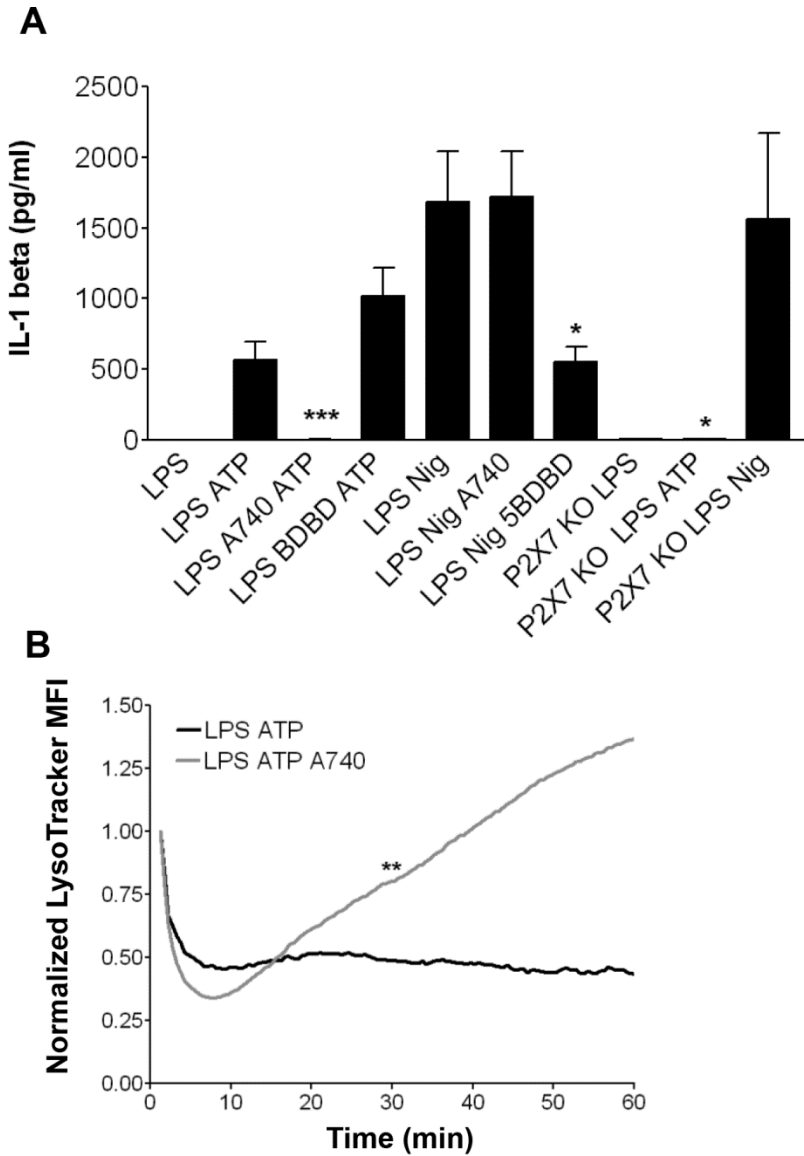
(A) WT BMDM were LPS-primed and ATP treated with or without 500  $\mu$ M MitoTEMPO. Cells were labeled with MitoTracker Red, imaged via live cell microscopy and assessed for MitoTracker loss. Data are presented as percent of cells/field retaining MitoTracker signal. Error bars represent mean  $\pm$ s.d. ( $n \geq 10$  fields; \*\*\*,  $P < 0.0001$ ). (B) WT BMDM were LPS-primed and treated with 3 mM ATP with or without 500  $\mu$ M MitoTEMPO. Cells were labeled with LysoTracker green, imaged via live cell microscopy and assessed for LysoTracker loss. LysoTracker green signal was recorded as percent of cells/field retaining signal at 0, 5, 10, 15, 30, 45, and 60 min after addition of ATP. Error bars represent  $\pm$ s.d. ( $n \geq 5$ , \*\*\*,  $P = 0.0008$ ). (C) WT BMDM were LPS-primed and treated with 3 mM ATP and 0  $\mu$ M, 100  $\mu$ M or 500  $\mu$ M MitoTEMPO. The amount of IL-1 $\beta$  present in cell supernatants was measured via ELISA. Error bars represent mean  $\pm$  s.d. ( $n \geq 2$ )

#### **4.5.5 The P2X7 receptor, but not the P2X4 receptor is required for ATP induced inflammasome dependent IL-1 $\beta$ secretion and organelle damage**

ATP activates the NLRP3 inflammasome through binding to the purinergic P2X7 receptor (Qu et al., 2007). We asked whether ATP-induced effects that we observed here were also P2X7 dependent. We tested the requirement for P2X7 in ATP-dependent IL-1 $\beta$  production using the P2X7 specific inhibitor A740003 (Riteau et al., 2012). We found that A740003 blocked the secretion of mature IL-1 $\beta$  in response to LPS priming and ATP treatment in WT BMDM, as detected via ELISA (Figure 4-4A). To confirm the specificity of the inhibitor, we also measured IL-1 $\beta$  secretion in P2X7<sup>-/-</sup> BMDM in response to LPS-priming and ATP stimulation. As expected, we saw that IL-1 $\beta$  secretion was abrogated in LPS primed P2X7<sup>-/-</sup> BMDM treated with 3 mM ATP, but not in LPS primed P2X7<sup>-/-</sup> BMDM treated with the potassium ionophore nigericin. These results indicate that P2X7 is required for ATP dependent activation of the NLRP3 inflammasome, but dispensable for activation via nigericin.

Since A740003 blocked IL-1 $\beta$  secretion, we next asked whether it affected the first NLRP3 dependent cellular effect we observed, LMP. We found that A740003 significantly reduced the number of cells that lost LysoTracker Green signal, indicating that P2X7 receptor was needed for ATP induced, NLRP3 dependent LMP (Figure 4-4B). Interestingly, we observed an initial loss of LysoTracker in the presence of A740003, but the cells recovered rapidly. These data show that the lysosomal damage could be reversible.

ATP can activate multiple purinergic receptors at different concentrations. We tested whether other purinergic receptors, such as P2X4, could also play a role in NLRP3 inflammasome activation. We found that the P2X4 inhibitor 5-BDBD (Chen et al., 2013; Hung et al., 2013) did not block mature IL-1 $\beta$  secretion from LPS primed WT BMDM treated with ATP (Figure 4-4A). Similarly, we found that 5-BDBD did not prevent LMP in LPS primed WT BMDM treated with ATP (data not shown). This shows that both the NLRP3 dependent secretion of IL-1 $\beta$  and LMP in response to ATP occur independently of P2X4. Surprisingly, we found a significant decrease in IL-1 $\beta$  secretion from LPS-primed WT BMDM treated with nigericin in the presence of 5-BDBD. These data suggest that P2X4 is required for NLRP3 inflammasome dependent IL-1  $\beta$  secretion in response to nigericin.



**Figure 4-4: P2X7 is required for ATP induced NLRP3 dependent events**

(A) WT or P2X7<sup>-/-</sup> BMDM were LPS-primed and stimulated with 3 mM ATP with or without 100 μM A740003 (labeled A740) or 50 μM 5-BDBD (5BDBD). IL-1β in supernatants was measured via ELISA. Error bars represent mean ± s.d. (n=6 for WT; n=3 for P2X7<sup>-/-</sup>; P=0.0013, \*\*\*, WT LPS ATP vs WT LPS ATP A740; P=0.0122, \*, WT LPS Nig vs WT LPS Nig BDBD; P=0.0197, \*, WT LPS ATP vs KO LPS ATP). (B) WT BMDM were LPS-primed, labeled with LysoTracker and stimulated with 3 mM ATP and with or without 100 μM A740003 at the start of live cell wide field imaging. Normalized LysoTracker mean fluorescence intensity after background subtraction is displayed (n≥3; P=0.0055, \*\*, at 30 minutes).

## 4.6 DISCUSSION

In this study, we found that ATP stimulation of the NLRP3 inflammasome causes NLRP3 dependent mitochondrial dysfunction, loss of mitochondrial and lysosomal membrane integrity, and secretion of IL-1 $\beta$ . Additionally, we found that following ATP stimulation, NLRP3 dependent LMP occurred prior to MMP, with only a subset of cells undergoing LMP also incurring mitochondrial damage. The organelle damage in response to ATP relied on mitochondrial ROS production, as inhibition of mitochondrial ROS abrogated MMP and delayed and reduced LMP. Finally, the ATP dependent, NLRP3 dependent IL-1 $\beta$  secretion and organelle damage required P2X7, but not P2X4. Unexpectedly, we also observed that P2X4 is required for nigericin induced IL-1 $\beta$  secretion in WT BMDM.

We have previously shown the importance of mitochondrial ROS for NLRP3 dependent IL-1 $\beta$  secretion, LMP, and MMP in response to nigericin. The results presented here further confirm that these effects are also more broadly applicable to other stimuli of the NLRP3 inflammasome. ATP stimulation of the NLRP3 inflammasome in LPS primed BMDM appears to be more sensitive to, or dependent on, mitochondrial ROS than nigericin induced NLRP3 activation. The mitochondrial inhibitor MitoTEMPO was able to more significantly inhibit IL-1 $\beta$  secretion, MMP, and LMP in ATP treated BMDM compared to nigericin treated BMDM. Furthermore, some reversal of LMP was seen after 45 minutes of ATP stimulation in LPS primed WT BMDM. These data suggest that without a threshold level of mitochondrial ROS being continually met in the presence of MitoTEMPO, lysosomes may be able to recover from their initial damage in response to ATP. This recovery was not observed in nigericin treated cells, possibly due to a greater amount of initial mitochondrial ROS production that could not be completely inhibited with 500  $\mu$ M MitoTEMPO. A second possibility is that nigericin induced

more direct damage to the mitochondria itself than did ATP. More importantly, these data suggest a role for mitochondrial ROS in perpetuating initial lysosomal damage in response to ATP or nigericin.

Unlike nigericin or pore-forming toxins, ATP has the potential to both act as a primary stimulus for NLRP3 inflammasome activation and to collaborate with other stimuli. ATP is always present in cells and can leak out, whereas nigericin and/or pore forming toxins are not present under most physiological conditions. We find that the addition of ATP alone to LPS primed cells resulted in NLRP3 inflammasome activation via the P2X7 receptor. However, nigericin, pore-forming toxins, and crystals (Chu et al., 2009; Heid et al., 2013; Riteau et al., 2012) can induce membrane damage or leakiness. ATP is also depleted from these cells (Walev et al., 1994), suggesting that it is secreted where it could engage P2X7 receptors and create a positive feedback mechanism of NLRP3 inflammasome activation (Gombault et al., 2012). There is a reduction of ATP levels in cells with an active NLRP3 inflammasome (Heid et al., 2013). However, whether this is due to leakage of ATP from the cell, or alteration of ATP production/degradation is unclear. Furthermore, any ATP leakage would potentially be lower than the 3 mM ATP needed for activation of NLRP3 via P2X7. Whether ATP leakage is sufficient to stimulate the NLRP3 inflammasome via P2X7 remains controversial. Although ATPases may degrade some released ATP, ATP dependent responses were found in THP-1 cells when activated with crystal NLRP3 agonists (Riteau et al., 2012). In fact, ATP levels in the supernatants correlated to IL-1 $\beta$  secretion (Riteau et al., 2012).

The P2X4 receptor is sensitive to lower concentrations of ATP than the P2X7 receptor (Raouf et al., 2007; Sun et al., 2013). While 3 mM ATP is optimal to activate the P2X7 receptor, there has been little study of its ability to activate the P2X4 receptor. Some groups have

suggested that the P2X4 receptor associates with the P2X7 receptor (Hung et al., 2013). Others have suggested that P2X4 acts as a regulator of P2X7, decreasing cellular ROS and limiting cellular damage (Kawano et al., 2012). Previous work in our lab has demonstrated that P2X4 is active only at lower concentrations of ATP, ranging from 0.01 mM to 1 mM (Sun et al., 2013). Our data here are consistent with the activity of the P2X7 receptor being sufficient for NLRP3 inflammasome dependent events to occur in LPS primed BMDM in response to 3 mM ATP. This may include a P2X7 dependent increase in cellular ROS required to perpetuate downstream NLRP3 inflammasome dependent events, such as organelle damage. Interestingly, we found that while the P2X4 receptor was not required for ATP induced inflammasome activation, P2X4 was required for nigericin induced inflammasome activation in WT BMDM. Nigericin could permit lower concentrations of ATP to leak from the cells or from the mitochondria, thus enabling cell-derived ATP to activate P2X4. At this lower concentration of ATP leakage induced by nigericin treatment, P2X4 could work to further perpetuate NLRP3 inflammasome dependent IL-1 $\beta$  secretion, LMP, and MMP. Although the mechanism of how P2X4 could accomplish this is unclear, one possibility is that its physical location on the lysosomal membrane could enhance lysosomal damage in response to nigericin induced, NLRP3-dependent cell damage and ATP leakage.

## 5.0 SUMMARY AND INTERPRETATIONS

The NLRP3 inflammasome is activated in response to a variety of diverse stimuli, initiating a complex series of events following its activation. Here, we have characterized two common NLRP3 inflammasome stimuli, nigericin and ATP. Using a combination of bulk biochemical assays and individual live cell microscopic analyses, we have proposed a sequence of events that occur during NLRP3 inflammasome activation. Our study has incorporated both previously reported NLRP3 dependent effects on the cell, as well as novel findings. More importantly, our work has assimilated and explained a variety of conflicting results observed with various NLRP3 stimuli under diverse conditions.

First, we investigated the interplay between organelles during NLRP3 activation. We found that nigericin-induced NLRP3 inflammasome activation resulted in NLRP3 independent mitochondrial dysfunction, including depolarization and decreased oxygen consumption rate, in murine bone marrow derived macrophages (BMDM). These events were then followed by NLRP3 dependent LMP, IL-1 $\beta$  processing and secretion, mitochondrial membrane permeabilization (MMP), loss of cell membrane integrity, and cell death. IL-1 $\beta$  processing and secretion, as well as LMP, were also seen in BMDM with TLO induced NLRP3 inflammasome activation. Furthermore, mitochondrial ROS were required for both lysosomal and mitochondrial damage, as well as processing and secretion of IL-1 $\beta$ . Our data suggest that NLRP3

inflammasome activation requires the production of ROS, lysosomal damage, and mitochondrial dysfunction.

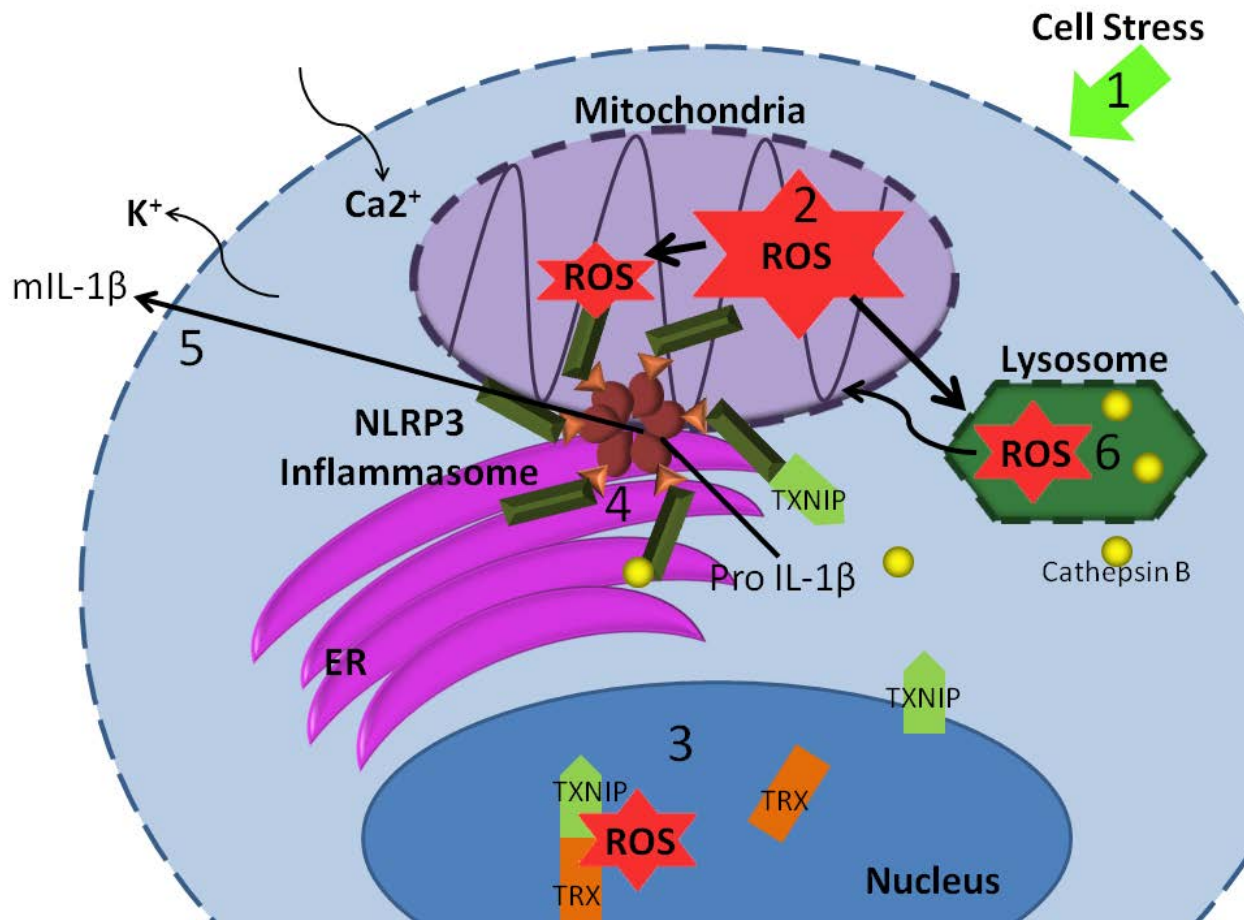
Next, we established a system for studying the inflammasome in a cell line. Using this system—D2SC-1 cells transduced with ASC—we found that ASC and caspase-1 were required in addition to NLRP3 for lysosomal membrane permeabilization (LMP), IL-1 $\beta$  processing and secretion following nigericin, ATP or TLO stimulation. This system successfully reconstituted the NLRP3 inflammasome because potassium efflux inhibited inflammasome activation, even though ASC was overexpressed in these cells. We used this system to demonstrate that the NLRP3 inflammasome is activated by electroporation. Taken together, these data indicate that NLRP3 inflammasome assembly and activation are regulated at a post-translational level.

Finally, we verified that the effects of NLRP3 inflammasome activation seen in response to nigericin were also observed during inflammasome activation via other stimuli. We found that ATP induced inflammasome activation resulted in the same cellular responses as nigericin stimulation in roughly the same sequence. IL-1 $\beta$  secretion, LMP, MMP, and mitochondrial dysfunction occurred in a NLRP3 inflammasome dependent manner. Mitochondrial ROS was also found to be required for MMP, IL-1 $\beta$  secretion, and the continuation of lysosomal membrane disruption. These data suggest that there is a greater dependence on mitochondrial ROS in response to ATP stimulation than nigericin stimulation of the NLRP3 inflammasome. However, the data could also be explained by higher levels of ROS production by nigericin than by ATP, and therefore, mitochondrial ROS inhibitors do not diminish NLRP3 inflammasome dependent responses as efficiently in nigericin treated cells. Although LMP still preceded MMP in ATP stimulated cells, both of these events occurred more rapidly than in nigericin treated cells. These data suggest that ATP treated BMDM die more rapidly following MMP than



BMDM stimulated with nigericin. This could explain the higher levels of IL-1 $\beta$  secretion seen in nigericin treated cells due to the fact these cells survive longer and therefore can process and secrete more cytokine.

From these studies, we propose a model which incorporates the kinetic information provided here with the knowledge of NLRP3 inflammasome activation in the field (Figure 5-1). First, we propose that cellular stress results in NLRP3 independent mitochondrial dysfunction and mitochondrial ROS production. This mitochondrial ROS then activates the NLRP3 inflammasome, possibly through redox sensitive proteins, such as TXNIP. NLRP3 inflammasome activation is dependent on potassium efflux and leads to the cleavage of pro-caspase 1 to generate active caspase-1, which then processes pro-IL-1 $\beta$  to mature IL-1 $\beta$ . Mature IL-1 $\beta$  is then secreted from the cell by an unknown mechanism. After the NLRP3 inflammasome is activated, cellular stress continues and more ROS is produced, possibly due to the limited ability of antioxidative proteins such as TRX, which is freed from TXNIP (Zhou et al., 2010). The lysosomal membrane undergoes NLRP3 inflammasome dependent LMP in response to a critical threshold level of ROS. Lysosomal disruption leads to more cellular ROS, as well as the release of lysosomal contents. The mitochondrial membrane is disrupted from this continued damage, as well as the potential opening of mitochondrial permeability transition (MPT) pores. This is quickly followed by cell death.



**Figure 5-1: Model of NLRP3 inflammasome activation in response to cell stress**

(1) Cell stress is induced by NLRP3 agonists such as nigericin or pore forming toxins, causing mitochondrial dysfunction and depolarization, and mitochondrial ROS generation (2). This causes TXNIP to dissociate from TRX (3), leading to NLRP3 inflammasome activation (4). This activation is dependent on potassium efflux and calcium influx. (5) Active NLRP3 processes and secretes mature IL-1β (mIL-1β). (6) Mitochondrial ROS damages lysosomes, leading to LMP and lysosomal ROS generation which acts back on the mitochondria leading to MMP. Cathepsin B is also released from lysosomes and could perpetuate NLRP3 inflammasome activation.

## 5.1 ROS

Reactive oxygen species have been proposed as a common mechanism of NLRP3 inflammasome activation (Zhou et al., 2011). Production of reactive oxygen species can occur independently of the NLRP3 inflammasome as a general consequence of cell stress (Brookes et al., 2004). Since most stimuli of the NLRP3 inflammasome contribute to cell stress, ROS is a common result of exposure to most NLRP3 inflammasome stimuli and is therefore an excellent candidate for the activator of NLRP3 (Dostert et al., 2008; Zhou et al., 2010). Our data support this idea of ROS as a key player in NLRP3 inflammasome activation. Initial mitochondrial disruption, characterized by loss of mitochondrial reductase activity, mitochondrial depolarization, decreased oxygen consumption rate, and decreased cellular ATP levels occur independently of the NLRP3 inflammasome, in response to an inflammasome stimulus, such as nigericin (Heid et al., 2013). Mitochondrial depolarization is linked to the production of ROS (Zhou et al., 2011). Following nigericin treatment of the BMDM, the resulting mitochondrial ROS may then activate the NLRP3 inflammasome if all components, including ASC, are present in the cell.

Following NLRP3 inflammasome activation, NLRP3 dependent effects in the cell commence. This creates a divergence in cell stress response between cells containing the active inflammasome components NLRP3, ASC, and functional caspase-1, and those cells lacking these components. Mitochondrial ROS, possibly in conjunction with the inflammasome complex directly, disrupts lysosomal membranes, resulting in LMP. Although this LMP can occur in the presence of an inhibitor of mitochondrial ROS, such as MitoTEMPO, it does not persist in response to ATP. Furthermore, in response to nigericin, WT BMDM show delayed LMP in the presence of MitoTEMPO. These results point to mitochondrial ROS as a key promoter of maintaining lysosomal membrane disruption. Another possible interpretation is that

mitochondrial ROS are required for continued loss of lysosomal acidic pH, which is detected by the lysosomal marker used, LysoTracker Green. As previously reported, IL-1 $\beta$  secretion is also inhibited in the presence of MitoTEMPO (Nakahira et al., 2011). This places the production of mitochondrial ROS upstream of both LMP and IL-1 $\beta$  processing and secretion.

The inhibition of IL-1 $\beta$  secretion in MitoTEMPO treated cells implicates the lysosome as a regulator of sustained inflammasome activation. LMP is a NLRP3 inflammasome dependent event, and therefore downstream of inflammasome activation. Although LMP initially occurs during ATP stimulation, in the presence of MitoTEMPO, IL-1 $\beta$  secretion is inhibited (Heid et al., 2013). In addition to mitochondria, lysosomes can be a source of ROS (Heid et al., 2013). Therefore, following LMP, lysosomes could release ROS, or indirectly lead to the production of ROS through lysosomal disruption or Fenton reactions, that perpetuate NLRP3 inflammasome activation, leading to significant levels of IL-1 $\beta$  secretion by BMDM. However, when MitoTEMPO decreases the amount of mitochondrially generated ROS, it also decreases lysosomal damage. This reduced lysosomal damage would result in reduced intracellular ROS. As a consequence, there may not be enough total ROS in the cell to drive continued NLRP3 inflammasome activation and IL-1 $\beta$  secretion.

Lysosomal ROS, or continued production of mitochondrial ROS, could lead to eventual breakdown of the mitochondrial membrane, or MMP. We have shown that MMP is also an NLRP3 inflammasome dependent event and always occurs following LMP within a subset of cells. When the production of mitochondrial ROS is blocked, MMP is reduced, possibly due to less direct mitochondrial damage and/or less or delayed ROS production from lysosomes. Following MMP, cell death occurs.

Although some reports suggest that the leakage of lysosomal proteases, such as cathepsin B, could activate the inflammasome (Hornung et al., 2008), the cathepsin B inhibitor only prevented IL-1 $\beta$  secretion in response to nigericin and not LMP or MMP. Therefore, cathepsin B could play a role in continued NLRP3 inflammasome activation, although NLRP3 dependent LMP and MMP still occur when the activity of cathepsin B is blocked. These data also support ROS as the initial activator of the inflammasome and necessary for continued organelle damage. Based on these data, we propose that a certain threshold level of ROS is required for continued NLRP3 inflammasome activation and its downstream effects following initial cell stress. Unfortunately, many studies only measure the presence of mitochondrial ROS with fluorescent dyes, such as MitoSOX (Nakahira et al., 2011). It is difficult to determine how much ROS of mitochondrial or lysosomal origin is required and when mitochondrial or lysosomal ROS are required for NLRP3 inflammasome activation. More detailed kinetic studies using a variety of both mitochondrial and cellular ROS specific dyes in combination with mitochondrial and cellular ROS inhibitors are needed.

One caveat when using MitoTEMPO and related mitochondrial ROS dyes is that they are not completely specific to mitochondrial generated ROS. Some of the compound remains in the cytosol, where it can scavenge cytosolic ROS. Furthermore, some compound leaks out of the mitochondria into the cytosol. This leakage is especially true when the membrane integrity of the mitochondria is compromised, as is the case during NLRP3 inflammasome activation. Therefore, MitoTEMPO could also be acting as an ROS scavenger for non-mitochondrial generated ROS within the cell. This caveat makes it difficult to determine if only mitochondrial ROS is driving NLRP3 inflammasome dependent events. The mitochondrial ROS indicator, MitoSOX, would also be able to detect cellular ROS, not specific to the mitochondria, further complicating this

issue. The depolarization of the mitochondria we observed does indicate that at least some mitochondrial ROS is generated in response to stimuli of the NLRP3 inflammasome, making the mitochondria at least one source of ROS in the cells. Furthermore, the inhibition of NLRP3 dependent IL-1 $\beta$  secretion, LMP, and MMP seen with MitoTEMPO also suggests involvement of mitochondrial ROS. Further study with other ROS scavengers not targeted to the mitochondria would need to be done to compare inhibition of NLRP3 inflammasome dependent events with inhibition seen with MitoTEMPO.

ATP activation of the NLRP3 inflammasome is dependent on the P2X7 receptor (Solle et al., 2001). We have shown that in addition to IL-1 $\beta$  processing and secretion, LMP is also dependent on P2X7. Interestingly, inhibition of P2X7 with a pharmacological antagonist, led to the recovery of initial LMP in WT BMDM. These results were similar to the recovery from LMP in the presence of MitoTEMPO in ATP treated cells. One possible explanation is that P2X7 activation is associated with ROS production in cells (Hung et al., 2013; Lenertz et al., 2009). Therefore, if P2X7, and any resulting pannexin pore, leads to increased ROS production, this could perpetuate ROS and NLRP3 dependent LMP and MMP. When P2X7 is inhibited, ROS production is also blocked (Hung et al., 2013) and the threshold level for continued LMP is not obtained. Therefore, downstream MMP also does not occur. However, we hypothesize that initial mitochondrial dysfunction, as measured by loss of mitochondrial reductase activity, decreased OCR, and mitochondrial depolarization, still occur in ATP treated BMDM when P2X7 is inhibited. This initial mitochondrial dysfunction would generate the ROS that cause temporary LMP.

During stimulation of the NLRP3 inflammasome, when direct damage to the lysosome occurs, such as the case of crystals, chemical lysotropic agents, or frustrated phagocytosis, it is

possible that initial ROS production by the mitochondria is not essential. Instead, direct and severe insult to the lysosome releases enough ROS and cathepsin B that the NLRP3 inflammasome is directly activated downstream of lysosomal damage, instead of upstream as with nigericin, ATP, and TLO. Cellular ROS is still required for crystal stimulation of the NLRP3 inflammasome, as ROS scavengers inhibit NLRP3 dependent IL-1 $\beta$  secretion (Dostert et al., 2008). Damage to the mitochondria may then occur following this initial lysosomal insult, rather than being the initial stimulus (Boya and Kroemer, 2008). Interestingly, the exposure time required for lysosome disrupting NLRP3 agonists to induce IL-1 $\beta$  secretion is much longer than the exposure time needed for NLRP3 agonists like nigericin, ATP, and TLO which initially activate the mitochondria (Dostert et al., 2008). This would suggest that lysosomal damage requires more time to generate the critical threshold level of ROS to fully activate the NLRP3 inflammasome.

## **5.2 P2X4**

P2X4 is another type of purinergic receptor expressed on endothelial cells, dendritic cells, macrophages, and microglial cells (Qureshi et al., 2007; Sakaki et al., 2013; Surprenant and North, 2009). It is sensitive to lower concentrations of ATP than P2X7 (Hung et al., 2013). Recently, P2X4 has been reported to interact directly with P2X7 and regulate its activity (Hung et al., 2013). Surprisingly, we found that P2X4 was required for nigericin induced NLRP3 inflammasome dependent IL-1 $\beta$  secretion, but not ATP-induced IL-1 $\beta$  secretion. Based on these data, if P2X4 does form complexes with P2X7, the latter is still the dominant purinergic receptor required for ATP-induced NLRP3 inflammasome activation and can compensate for P2X4

inhibition. However, in nigericin-induced NLRP3 inflammasome activation, P2X7 is not required and therefore, the more subtle effect of P2X4 can be observed.

Intracellular ATP levels decrease in response to nigericin treatment in WT BMDM. It is possible that ATP leaks out of these cells, since they lose cell membrane integrity and leak LDH (Heid et al., 2013). Furthermore, the dysfunctional mitochondria could also be leaking ATP locally into the intracellular environment. P2X4 has been reported on both the surface of cells, as well as on lysosomal compartments (Qureshi et al., 2007). Therefore, P2X4 receptors at either location could be activated by lower ATP concentrations that are insufficient to activate P2X7.

Studies that demonstrated lower levels of ATP are insufficient to activate P2X7 could have been activating P2X4 (Qu et al., 2007). However, unlike nigericin treatment, 1 mM ATP treatment did not produce optimal amounts of IL-1 $\beta$  secretion. If both stimuli act through P2X4, this indicates an intrinsic difference between stimuli or in the location where P2X4 is activated. Interestingly, lower levels of ATP were found to induce more cathepsin B release from cells than higher levels of ATP in a calcium dependent manner (Qu et al., 2007). This further implies a role for P2X4 on lysosomes leading to LMP. Thus, a role for P2X4 involvement in LMP is a new area for future research.

P2X4 has been reported to help control P2X7 dependent ROS levels in the cell (Kawano, 2012). In ATP treated cells, P2X4 activation reduces P2X7 induced ROS and suppresses autophagy (Kawano et al., 2012). In nigericin treated cells, the mitochondrial ROS generated likely does not require P2X7, since P2X7<sup>-/-</sup> BMDM display normal NLRP3 inflammasome activation. Therefore, the role of P2X4 in mediating this nigericin induced ROS is unknown. However, P2X4 activation during nigericin-induced NLRP3 inflammasome activation could inhibit the turnover of damaged mitochondria via autophagy (mitophagy). An accumulation of



damaged mitochondria could result in more mitochondrial ROS production in a P2X4 dependent manner in nigericin treated BMDM. If P2X4 is inhibited, damaged mitochondria could be removed faster, which would prevent the critical threshold of ROS production. Failure to meet this threshold would inhibit the NLRP3 dependent IL-1 $\beta$  secretion. Our findings have opened new avenues of research examining the role of P2X4 and ROS. Further studies using P2X4 receptor antagonists and ROS specific dyes could be conducted to explore the possible reduction in ROS levels in the absence of P2X4 activity, and compare nigericin and ATP treated cells.

### 5.3 LOCALIZATION

The NLRP3 inflammasome has been shown to assemble at the ER/mitochondria interface (Misawa et al., 2013; Park et al., 2013). This provides a mechanism for direct access of mitochondrial ROS to the NLRP3 inflammasome complex (Veal et al., 2007; Zhou et al., 2011). As our studies have shown, this initial wave of mitochondrial ROS is imperative for inflammasome activation. Although it is possible that ROS directly interact with NLRP3, they may also act through an intermediate. One possible intermediate is TXNIP, which can activate the NLRP3 inflammasome (Zhou et al., 2011). Studies have suggested that TXNIP may be a direct binding partner of NLRP3 (Zhou et al., 2011). ROS leads to the dissociation of TXNIP with thioredoxin (TRX), which would free TXNIP to activate NLRP3. In this model, ROS production would remain a critical step. TXNIP can also bind to Trx2, which is located in the mitochondria (Saxena et al., 2010). In a ROS-dependent manner, NLRP3, ASC, TXNIP, and Trx2 all localize to the mitochondrial membrane, which is a potential source of ROS. Moreover, the production of ROS has been shown to be conducive for MPT pore opening (Scorrano et al.,

1997). This pore complex involves voltage-dependent anion channels (VDAC), which are required for NLRP3 inflammasome activation (Tsujimoto and Shimizu, 2007; Zhou et al., 2011). These channels can form at the mitochondrial/ER interface and allow for calcium ion exchange into the mitochondria (Zhou et al., 2011). Furthermore, calcium flux has been reported to be critical for NLRP3 inflammasome activation (Murakami et al., 2012). The close proximity of the NLRP3 inflammasome to the mitochondria also suggests that the inflammasome may directly interact with the mitochondrial membrane to induce final membrane breakdown.

Although there is no evidence that lysosomes directly interact with the NLRP3 inflammasome, it is possible that lysosomes are not physically far apart from the mitochondria. Therefore, it is possible that mitochondrial produced ROS reach the lysosomes. This spatial separation may explain why a critical threshold level of ROS levels in the cell must be reached for LMP to occur and be maintained. Mitochondrial ROS would need to continually diffuse to lysosomes to induce and maintain LMP. Perhaps the natural ROS scavenging ability of the cell must be overcome for mitochondrial ROS to reach the lysosomes.

Although speculative, it is possible that lysosomes are brought to the site of NLRP3 inflammasomes during phagolysosome formation and membrane turnover. NLRP3 inflammasomes are ubiquitinated, marked for degradation, and transported to lysosomes for degradation in an attempt to control inflammasome activation (Shi et al., 2012). Furthermore, damaged mitochondria that arise during NLRP3 inflammasome activation must be removed. Autophagosomes associate with the NLRP3 inflammasome (Hamasaki et al., 2013), most likely in an attempt to degrade inflammasome complexes. In order for lysosomes to fuse with autophagosomes, SNARE proteins are needed (Kramer, 2013). One candidate SNARE for this fusion is Syntaxin 17 (Hamasaki et al., 2013). Interestingly, this SNARE is enriched at ER-

mitochondrial contact sites (Hamasaki et al., 2013; Itakura et al., 2012). Therefore, it is possible that lysosomes move along the microtubule toward the NLRP3 inflammasome to aid in phagolysosome formation. This also presents a possible mechanism for the secretion of IL-1 $\beta$  in vesicles from the cell.

#### 5.4 SIGNIFICANCE

Numerous diseases have been associated with activation of the NLRP3 inflammasome and excessive induction of inflammatory cytokines, such as IL-1 $\beta$ . These diseases include inflammatory bowel disease, gout, arthritis, cryopyrin-associated periodic syndrome (CAPS) and Muckle-Wells syndrome, among many others (Gabay et al., 2010). Typically, the treatment for these diseases involve blocking of the IL-1 receptor with pharmaceutical reagents, such as anakinra (Mertens and Singh, 2009). Although effective, this does not prevent NLRP3 inflammasome activation or any of the other downstream effects on organelles. Blocking the IL-1 receptor only blocks one result of NLRP3 inflammasome activation, without addressing the initial problem – unregulated activation of the NLRP3 inflammasome and ROS production.

From the data we have shown, we know that mitochondrial dysfunction, LMP, and cell death are also occurring in macrophages and dendritic cells as a result of the active NLRP3 inflammasome. These events lead to more cell damage and inflammation and potential damage to bystander cells. Our data support a proposed role for ROS production as a critical step in NLRP3 inflammasome activation. ROS scavengers and inhibitors have widespread effectiveness *in vitro*, preventing or reducing NLRP3 inflammasome activation and all of its known downstream effects, including IL-1 $\beta$  secretion (Dostert et al., 2008; Heid et al., 2013). ROS is

also a common requirement for most known NLRP3 inflammasome stimuli, making it a practical target for clinical applications (Dostert et al., 2008; Zhou et al., 2010). Since our data support the idea that mitochondrial ROS are critical for continued NLRP3 inflammasome activation and downstream effects, mitochondrial ROS scavengers would be a potential therapy for patients with NLRP3 inflammasome disorders such as Muckle-Wells syndrome or CAPS. Although MitoTEMPO is typically used *in vitro*, a similar reagent, MitoQ is a mitochondrial ROS scavenger that has been used *in vivo* in mouse studies (Dashdorj et al., 2013). MitoQ effectively reduces ROS production, NLRP3 inflammasome activation, and subsequent IL-1 $\beta$  and IL-18 release in a murine colitis model (Dashdorj et al., 2013).

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