STUDIES ON REDOX MODULATION OF NLRP3 INFLAMMASOME

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Abstract

Studies on Redox Modulation of NLRP3 Inflammasome

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NLRP3 inflammasome activation provides an innate host defence against pathogens. Two steps of inflammasome complex activation are recognised: Signal 1 or priming (expression of NLRP3 protein, pro-IL-1β, pro-IL-18 and pro-caspase-1), followed by Signal 2 or activation (expression of active IL-1β and IL-18). Additionally, caspase 5 may be involved *via* a non-canonical pathway. The purpose of this study was to investigate the possible role of cytosolic superoxide and slow-release hydrogen sulfide generation in mitochondria, in activating the NLRP3 inflammasome in THP-1 and HUVEC and EA.hy926 endothelial cells. Cells were differentiated with PMA (5 ng/ml) for 1-3 days. Subsequently, LPS treatment (5 µg/ml) was administered for 24 h for Signal 1 activation followed by bzATP (300 µM) for 1 hour for Signal 2. Signal 1 and Signal 2 of the NLRP3 inflammasome were up-regulated in the human macrophage cell model. Both endothelial cell types showed evidence of low level Signal 1 activation. Despite HUVEC cells showing priming of the NLRP3 inflammasome, there was no evidence for Signal 2 activation. Surprisingly, EA.hy926 cells showed low levels of active IL-1β, when exposed to PMA, suggesting other endpoints of an active NLRP3 inflammasome such as HMGB1, which was expressed in EA.hy926 cells, are worthy of investigation. Subsequently, the effects of intracellular generation of superoxide (mitoparaquat and paraquat at 1 and 5 µM) were investigated in differentiated THP-1 cells before and after LPS. In our hands, intracellular superoxide generation was unable to enhance Signal 1 NLRP3 inflammasome in PMA-differentiated THP-1 cells. However, the higher concentration of mitoparaquat was able to increase Signal 2. Non-canonical pathway was not detected in THP-1 cells. Interestingly, slow-release hydrogen sulfide donors (GYY4137 and AP39) reduced Signal 2 NLRP3 inflammasome suggesting anti-inflammatory properties. These data support a role for redox active oxygen and sulfur species in modulating inflammatory IL-1β and IL-18 synthesis in human monocytes with pro- and anti-inflammatory activities respectively.

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Table of contents

Table of tables

Table of figures

List of Abbreviations

α-KG	α-ketoglutarate
3MP	3-mercaptopyruvate
3MST	3-mercaptopyruvate sulfurtransferase
ACR	Accumulation ratio
ACTB	β-actin
AIF	Apoptosis-inducing factor
AIM2	Absent in melanoma 2
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	5' adenosine monophosphate-activated protein kinase
AOAA	Aminooxyacetic acid
AP39	10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide
ASC	Apoptosis-associated speck-like protein containing a CARD
APS	Ammonium persulfate
ATF-2	Activating Transcription Factor 2
ATP	Adenosine Triphosphate
BCA	β-cyano-L-alanine
BIR	Baculovirus inhibitor of apoptosis repeat
BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
bzATP	2',3'-(4-benzoyl)-benzoyl-adenosine triphosphate
CARD	Caspase activation and recruitment domain
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthase
CSE	Cystathionine γ-lyase
CO	Carbon monoxide
CD	Cluster of differentiation
CDO	Cysteine dioxygenase
CL	Cysteine lyase
CRP	C-reactive protein
Cu^{2+}	Cupric
Cu^{+}	Cuprous
CXCL16	Chemokine (C-X-C motif) ligand 16
DAMP	Danger-associated molecular pattern
DAPK	Death-associated protein kinase
DCM	Diabetic cardiomyopathy
DMEM	Dulbecco’s modified eagle medium
DRP 1	Dynamine related protein 1
DTT	1,2-dithiole-3-thiones
ECE	Endothelin-converting enzyme
EDTA	Ethylenediaminetetraacetic acid
EDRF	Endothelium-derived relaxing factor
Egr-1	Early growth response protein 1
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPCR	Endothelial cell protein c receptor

ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERMES	Endoplasmic reticulum-mitochondria encounter structure
ET	Extracellular trap
ETC	Electron transport chain
Ets-1	E26 transformation-specific-1
Fas	First apoptosis signal receptor
FCCP	Carbonyl cyanide p-(tri-flluromethoxy) phenyl-hydrazone
GPI	Glycophosphatidylinositol
Grb2	Growth factor receptor-bound protein 2
GYY4137	Morpholin-4-ium 4 methoxyphenyl (morpholino) phosphinodithioate
H_2S	Hydrogen sulfide
H_2O_2	Hydrogen peroxide
HATs	Histone acetyltransferases
H-CAT1	Human cationic amino acid transporter
HCL	Hydrogen chloride
HDAC	Histone deacetylases
HIF-1α	Hypoxia inducible factor-1α
HMEC-1	Human dermal microvascular endothelial cells
HMGB1	High mobility group box 1
HO-1	Heme oxygenase 1
HSP-27	Heat shock protein 27
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
ICE	IL-1β-converting enzyme (caspase-1)
IFN-γ	Interferon gamma
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK4	Interleukin-1 receptor-associated kinase 4
IRE1α	Inositol-requiring enzyme-1α
LDL	Low-density lipoprotein
LED	Light Emitting Diodes
LOX1	Lectin-like ox-LDL receptor-1
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTP	Long-term potential
MAPK	Mitogen-activated protein kinases
MAVS	Mitochondrial antiviral-signaling protein
MCDU	Human mercaptolactate-cysteine disulfiduria
MHC	Major histocompatibility complex
MIP2	Macrophage inflammatory protein 2
MitoPQ	Mitoparaquat
MPO	Myeloperoxidase
mPTP	Mitochondrial permeability transition pore
MPA	Mycophenolic acid
MtDAMPs	Mitochondrial DAMPs

MtDNA	Mitochondrial DNA
MtROS	Mitochondrial ROS
NAC	N-acetyl-L-cysteine
NaCl	Sodium chloride
NADH	Reduced nicotinamide adenine dinucleotide
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide-binding domain
Nck1	Non-catalytic region of tyrosine kinase adaptor protein 1
NET	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOD	Nucleotide-binding and oligomerization domain
NOX	NAD(P)H oxidase
Nrf2	Nuclear factor-like 2
NSAIDs	Non-steroidal anti-inflammatory drugs
PAG	D,L-propargylglcine
PAMP	Pathogen-associated molecular pattern
PARP1	Poly(ADP‑ribose) polymerase 1
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
PKC	Protein kinase C
PKR	Double-stranded RNA-dependent protein kinase
PMA	Phorbol 12-myristate 13-acetate
PMAr	Resting PMA
PQ	Paraquat
PR3	Proteinase 3
PTP	Permeability transition pore
PVDF	Polyvinylidene fluoride
PYD	Pyrine domain
qPCR	Quantitative polymerase chain reaction
RAGE	Receptor for advanced glycation end products
RCF	Relative centrifugal force
RIPA	Radioimmunoprecipitation assay
RIRR	Reactive Oxygen Species (ROS)-induced ROS-release
RLR	Retinoic acid-inducible gene–I-like receptor
ROS	Reactive oxygen species
RPL37A	Ribosomal protein L37a
RPM	Revolutions per minute
RT-PCR	Real time polymerase chain reaction
SEM	Standard error of the mean
SESN2	Sestrin 2
SDS	Sodium dodecyl sulphate
SMAC	Second mitochondria-derived activator of caspase

SOD	Superoxide dismutase
SQR	Sulfide quinone oxidoreductase
STAT1	Signal transducer and activator of transcription 1
TBS-T	Tris-buffered saline tween-20
TCA	Tricarboxylic acid
TCBQ	Tetracholorobenzoquinone
TEMED	N, N, N', N'-Tetramethylethylenediamine
TF	Tissue factor
TGF-β	Transforming growth factor beta
Th	T-helper
TIR	Toll/interleukin 1 (IL-1) receptor
TNF-α	Tumour necrosis factor alpha
TLR	Toll-like receptors
TMAO	Trimethylamine N-oxide
TPP	Triphenylphosphonium
TPPB	2-[2-(triphenylphosphonio)ethyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1benzopyran-6-ol bromide
Trx	Thioredoxin
TXNIP	Thioredoxin-interacting protein
UTP	Uridine-5'-triphosphate
VCAM-1	Vascular cell adhesion molecule-1
VD3	1, 25-dihydroxyvitamin D
XO	Xanthine oxidase

Chapter 1 Introduction

1.1 Innate immunity

The immune system is divided into innate and adaptive branches. The innate immune system appeared early in evolutionary terms and is mediated by the germ-line, hence it is genetically encoded. Activation of adaptive immune system occurs after the innate component (Medzhitov and Janeway 2000) (**Figure 1-1**).

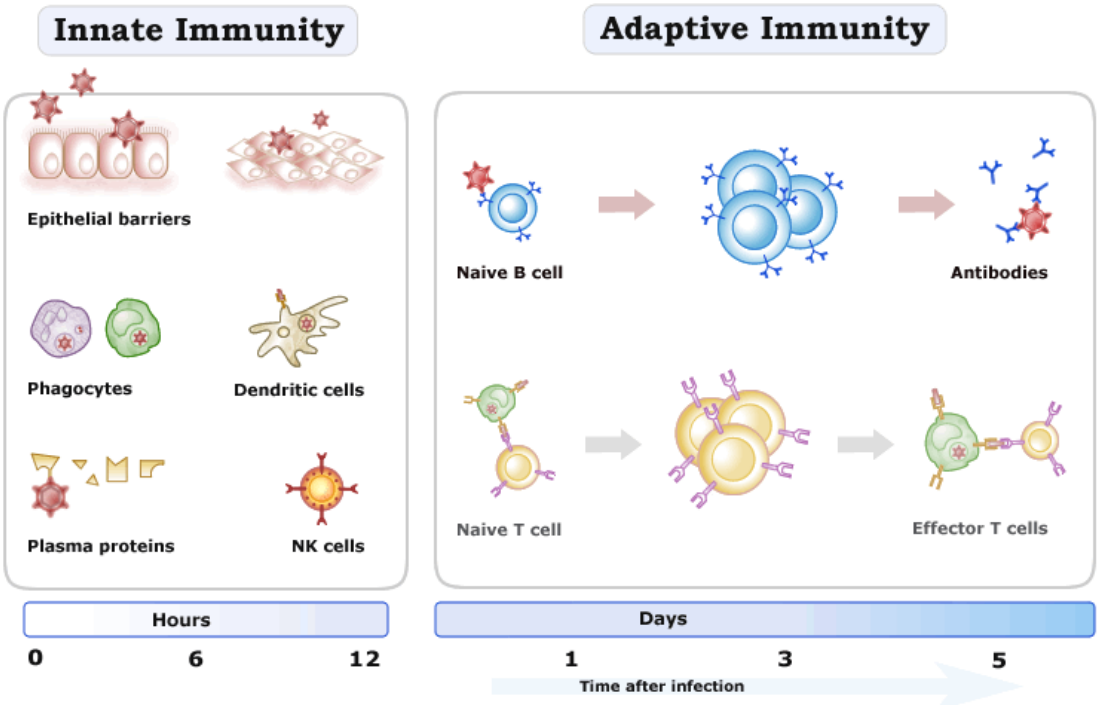

Figure 1-1 The main components of innate and adaptive immunity.

Schematic diagram shows the differences between innate and adaptive immunity with respect to how quickly the response occurs to pathogens and its central effector cell types (Abbas et al., 2014).

The innate immune system involves the combined actions of epithelial barriers, plasma protein activation as well as a variety of cell types including monocyte-macrophages and makes an immediate response to pathogens within hours (Iwasaki and Medzhitov, 2010). Furthermore, endothelial cells are also involved in innate immunity pathways which will be further elaborated in this study.

Pattern-recognition receptors (PRR) are the key components of the innate immune response and will recognise the signals such as pathogen-associated molecular patterns (PAMPs), produced following infection by pathogens, or damage associated-molecular patterns (DAMPs) released during trauma, ischaemia or essentially any form of tissue/cellular damage (Kankkunen et al., 2010) (Land, 2013) (**Figure 1-2**). Additionally, PRR are involved in producing the major histocompatibility (MHC)-molecule complex by the adaptive immune system (Imler and Hoffmann, 2000).

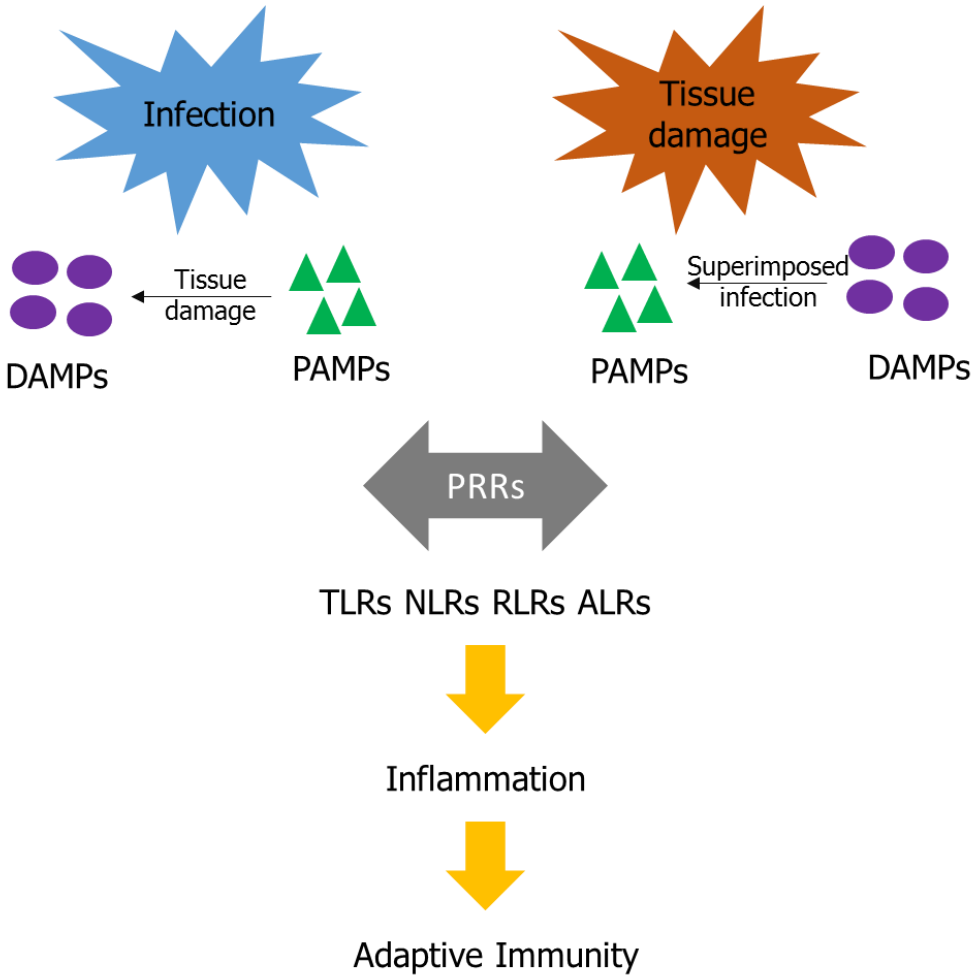

Figure 1-2 Types of signal recognised by pattern recognition receptor (PRR).

Signalling receptors recognize PAMPs and DAMPs hence activate signal-transduction pathways that induce the expression of inflammatory cytokines and regulate the adaptive immune response, RLR: RIG-1 like receptor (Land, 2013).

These receptors include nucleotide binding oligomerization domain (NOD)-like receptor (NLR), Toll-like receptor (TLR) and 'Absent in Melanoma 2' (AIM2)-like receptor (ALR) (Fukata et al., 2009). NLR and ALR sense intracellular signals from pathogens in the cytoplasm, whereas TLR sense extracellular signals at the cell surface or within endosomes for upregulation of inflammasome components (**Figure 1-3**). In view of rarity of TLR gene polymorphism, NLR has been of interest to study in recent years (Becker and O'Neill, 2007).

Excessive or insufficient activation of the innate immune response leads to a variety of inflammation related disease such as asthma, allergy, autoimmunity and arthritis, hence study of balancing the immune response is important for further new therapeutic opportunities (Elliott et al., 2014).

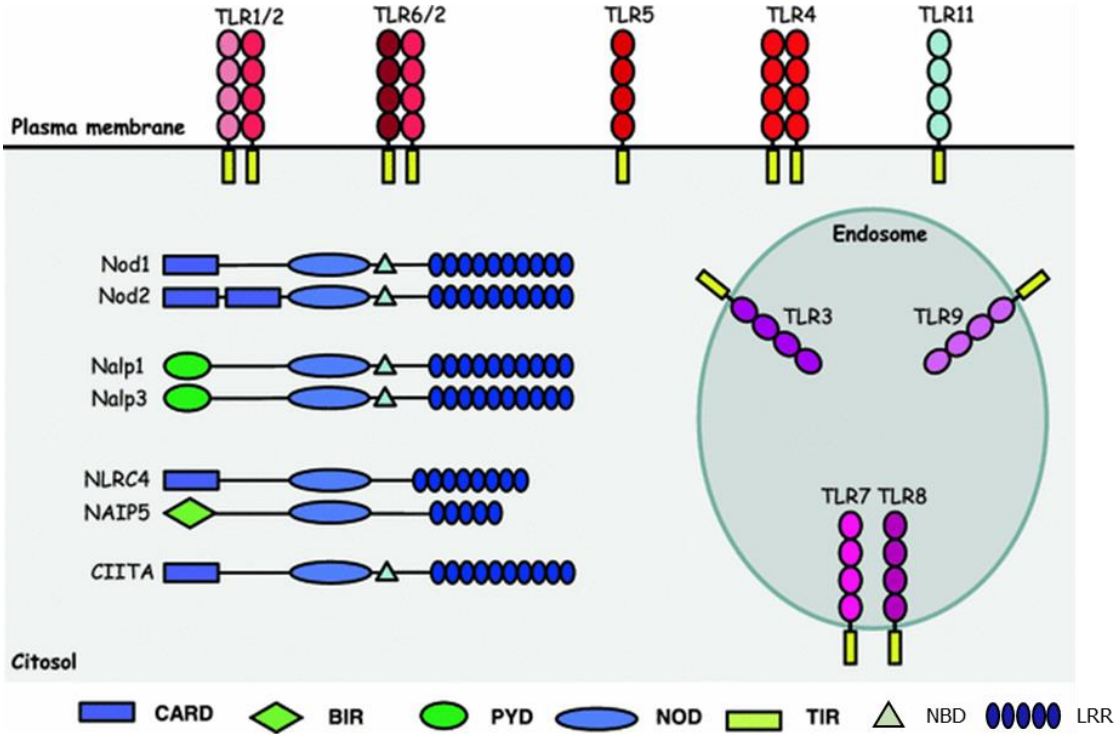

Figure 1-3 The cellular location of TLR and NLR.

TLR is a transmembrane protein which is located on the plasma membrane or endosome, whereas NLR is a cytosolic protein initiating the inflammatory response or pyroptosis. CARD: caspase activation and recruitment domain, BIR: baculovirus inhibitor of apoptosis repeat, PYD: pyrin domain, NOD: nucleotide-binding oligomerization domain, TIR: Toll/interleukin 1 (IL-1) receptor, NBD: nucleotide-binding domain and LRR: leucine-rich repeat. Adapted from (Bortoluci and Medzhitov, 2010).

1.2 Inflammasomes and pyroptosis

Inflammasomes are intracellular multiprotein complexes widely studied for the past fifteen years (Man and Kanneganti, 2015). An inflammasome is assembled in response to recognition of PAMPs and DAMPs; this priming process is widely known as 'Signal 1'. An activation signal (Signal 2) is then required for the biological activity associated with the inflammasome, namely maturation of IL-1β and IL-18. Furthermore, inflammasome activation results in a pro-inflammatory form of cell death known as pyroptosis (Bortoluci and Medzhitov, 2010) (**Figure 1-4**).

Pyroptosis is caspase-1 dependent and most frequently will be triggered by DAMPs or PAMPs. Although pyroptosis is considered to be a form of programmed cell death, it is distinct from the immunological cell death presented by apoptosis or cell necrosis. Pyroptosis is accompanied by plasma membrane rupture, H_2O influx, cellular swelling, cell lysis and release of pro-inflammatory cellular cytokines. Pyroptosis is also accompanied by DNA fragmentation and nuclear condensation which is distinct from DNA laddering characteristic of apoptosis, as the nuclear integrity is well maintained. However, the regulation of pyroptosis is not definite (Schroder and Tschopp, 2010). Pyroptosis is initiated by oligomerized ASC (apoptosis-associated speck-like protein containing a CARD), pyroptosome which rapidly activates caspase-1 and the release of inflammatory cytokines (Fernandes-Alnemri et al., 2007). **Table 1-1** summarizes the different cell morphologies, mechanisms and outcomes of the three forms of cell death.

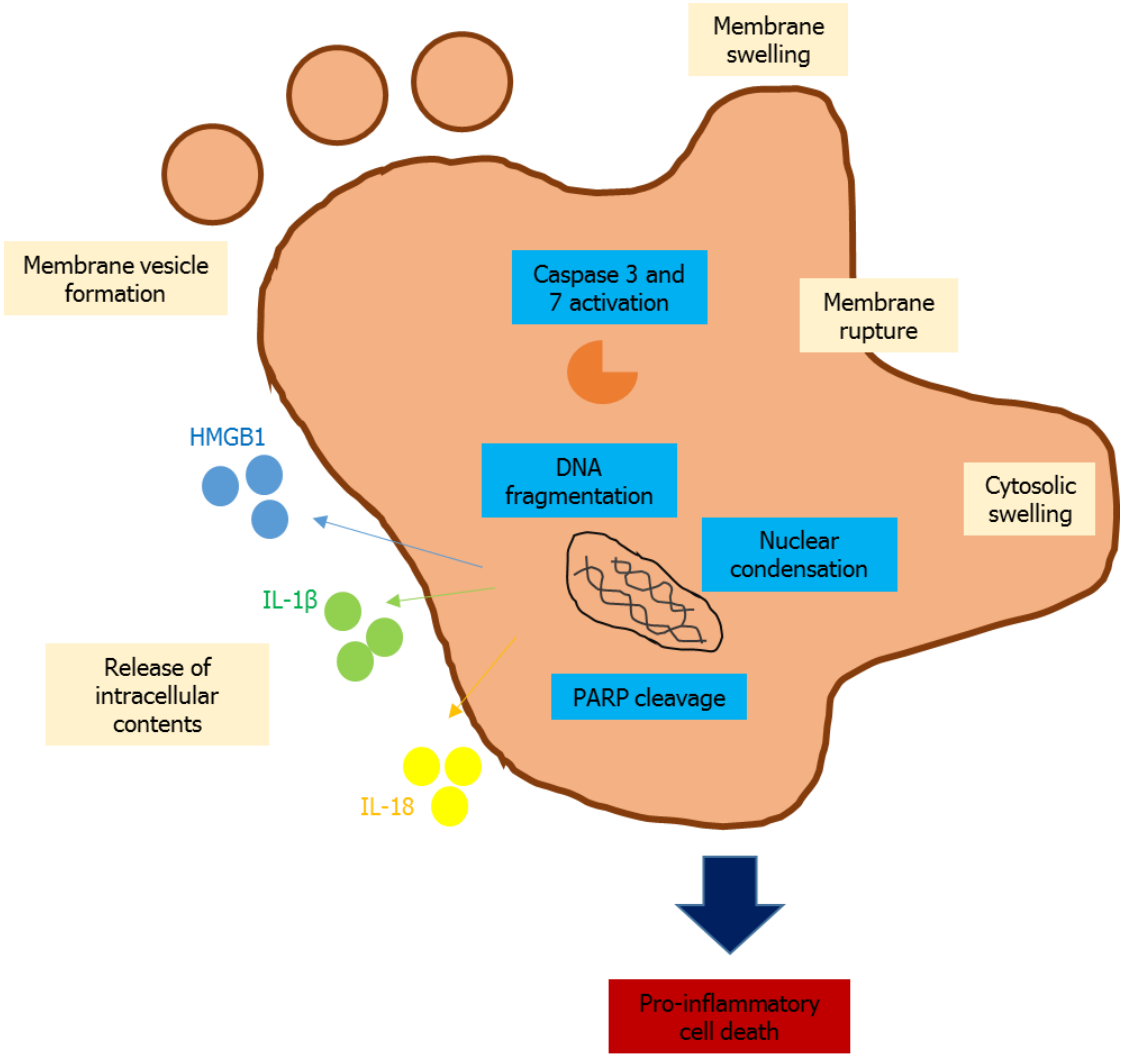

Figure 1-4 The process of cell pyroptosis.

Morphologically, pyroptotic cells are characterized by the early loss of plasma membrane integrity and this is accompanied by the shedding of membrane vesicles. Pyroptotic and apoptotic cells share several prominent features (shown in blue boxes). However, the volume of the cytoplasmic compartment of pyroptotic cells increases, whereas apoptosis is characterized by general shrinkage of the cell volume. HMGB1: High mobility group box 1 protein and PARP1: poly(ADP‐ribose) polymerase 1. Adapted from (Lamkanfi, 2011).

	Characteristics	Pyroptosis	Apoptosis	Necrosis
Morphology	Cell lysis	Yes	No	Yes
	Cell swelling	Yes	No	Yes
	Pore formation	Yes	No	Yes
	Membrane blebbing	Yes	Yes	No
	DNA fragmentation	Yes	Yes	Yes
Mechanism	Caspase-1	Yes	No	No
	Caspase-3	No	Yes	No
	Cytochrome *c* release	No	Yes	No
Outcome	Inflammation	Yes	No	Yes
	Programmed cell death	Yes	Yes	No

Table 1-1 Three forms of mammalian cell death.

Summaries of the different morphologies, mechanisms and outcomes of cell deaths.

The basic unit of the inflammasome complex consists of three components; an upstream sensor or receptor, the downstream adaptor and the effector (**Figure 1-5**). The receptors are either NLR or ALR. Apoptotic speck-like protein containing caspase-1 activation and recruitment domain, CARD (ASC) constitutes the adaptor and procaspase-1 forms the effector molecule (Atianand et al., 2013). Activation of caspase-1 leads to the cleavage of the pro-forms of inflammatory cytokines, namely, pro-IL-1β to lL-1β and pro-IL-18 to IL-18, which would activate the inflammation cascade.

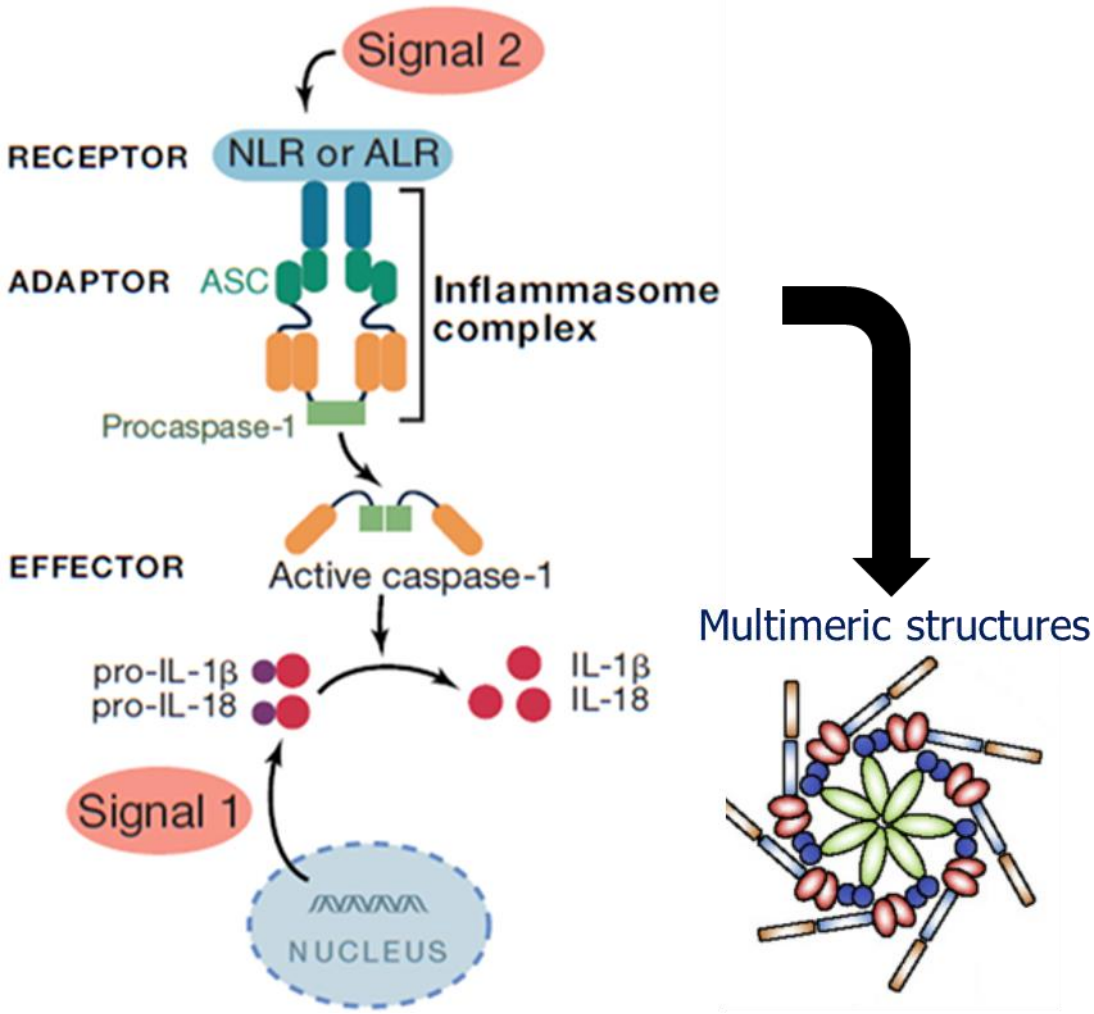

Figure 1-5 The basic inflammasome multiprotein complex.

The basic monomeric structure of an inflammasome unit consists of a sensor protein, an adaptor and an effector. Multimeric structures as illustrated are believed to be the active forms of the inflammasome. Adapted from (Atianand et al., 2013).

There are at least 22 NLRs, however NLRP1, NLRP3, NLRP6, NLRP7, NLRP12 and NLRC4 are the type of sensors that create the inflammasome (Lu and Wu, 2015). In addition, the non-NLR proteins, ALR and RIG-I (retinoic acid-inducible gene 1)-like receptors are also involved in inflammasome complexes (Kanneganti, 2015).

Infection can launch a 'self-destruct' and warning system in the host cell. Two types of receptors that belong to different families of PRRs which can sense intracellular and extracellular 'danger' signals are NLRs and TLRs (Yin et al., 2009b). The 'danger' signals can be given off by invasive pathogens, or by an injury to a tissue, which all can be recognised by the host cells receptors. This recognition will determine the fate of the host cell by a distinct mechanism; it will induce either the production of inflammatory chemical messengers termed 'cytokines' or programmed cell death (Kankkunen et al., 2010). Commonly found cytokines are tumour necrosis factor (TNF), IL-6, IL-8, type I interferons (IFNs) and Interferon regulatory factor (IRFs).

As mediators of the acute phase of inflammation, the cytokines are extremely important for the immune system to react to invading pathogens. It is however equally important for inflammasomes to distinguish pathogenic from non-pathogenic commensals, implying that a disturbance in normal danger signalling through the inflammasome can act as a master switch between tolerance and sensitization in many tissues (Davis et al., 2011). The activation of inflammasomes in myeloid innate immune cells plays an important role in acute and chronic inflammatory diseases and has been studied widely (Peeters et al., 2015).

1.3 NLRP3 inflammasome and its role in disease

NLR comprises three main domains: the C-terminal leucine-rich repeat (LRR) domain, a central domain and the most important N-terminal domain (Lechtenberg et al., 2014). The C-terminal repeat domain consists of a leucine-rich repeat (LRR) domain and the central unit has a nucleotide-binding and oligomerization domains (NACHT). Both are known to inhibit the NLR sensor in the absence of a trigger (Hu et al., 2013).Furthermore, the N-terminal domain includes a pyrin domain (PYD), CARD or a baculoviral IAP repeat domain (BIR) (Ting et al., 2008) **(Figure 1-6)**. NLRP3 inflammasome is so-called because its Nod-like receptor protein contains pyrin domain 3 (Elliott and Sutterwala, 2015). NLRs are specific sensors within the cytosolic compartment and are involved in amplifying the immune response and in pyroptosis (Sagulenko et al., 2013).

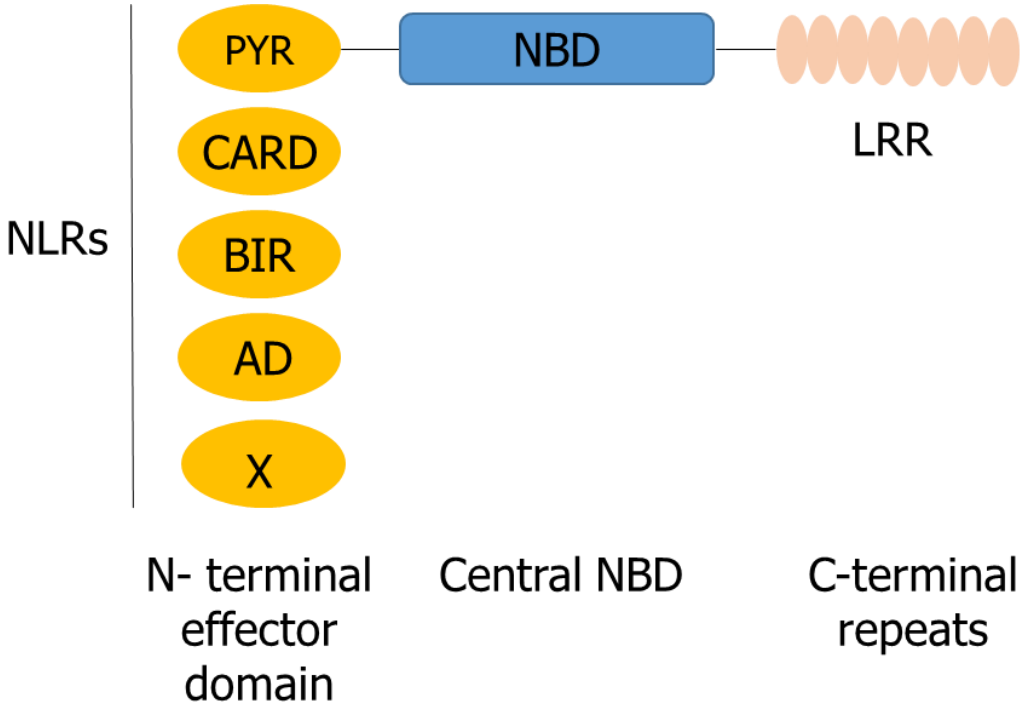

Figure 1-6 NLR domains

The NLRP3 gene provides instructions for making a protein called cryopyrin or NLR protein. NLR proteins are defined by three characteristics: an N-terminal effector domain, a central NBD (nucleotide-binding domain) and C-terminal repeats. When defined, NLR effector domains consist of either a PYR, CARD, BIR or a transactivation domain (AD). One NLR protein has an undefined or uncharacterized effector domain (X). (Ting et al., 2008).

NLRP3 is inactive in its monomeric form. It needs attachment of CARD from the adaptor protein (ASC) and CARD from the effector protein (caspase) to form the oligomerized structure (CARD-CARD). The CARD domains bring protein monomers of pro-caspase-1 into close proximity. This structure is known as NLRP3 inflammasome complex (Davis et al., 2011). Furthermore, this structure will initiate pro-caspase-1 self-cleavage and formation of the active caspase-1. Active caspase-1 triggers proteolytic cleavage of pro-IL-1β and pro-IL-18 (**Figure 1-7**) to yield active interleukins which mediate the immune response (Martinon et al., 2002).

Several theories have been proposed to account for the activation of the NLRP3 inflammasome. Potassium efflux, for example, is induced by extracellular ATP or following bacterial toxin pore formation through P2X7 receptor (Kahlenberg and Dubyak, 2004). Furthermore, engulfed PAMPs and DAMPs induce the formation of phagosomal vesicles and the ruptured vesicles will activate the NLRP3 inflammasome by release of reactive oxygen species (ROS), loss of phagosomal acidity and release of lysosomal calcium and cathepsin-B (Hornung et al., 2008).

Until now, the precise mechanisms and pathways of regulating the NLRP3 inflammasome remain unclear but as with all inflammasomes a “priming” signal and second “activation” signal are required (Lin et al., 2014). The priming signal of NLRP3 inflammasome which is typically intracytosolic enhances gene expression of precursor pro-interleukin (IL)-1β and NLRP3 *via* transcription factor nuclear factor-kappa B (NF-κB). Next, the activation signal promotes the organization of inflammasome components and their assembly into a multimer as in **Figure 1-5** (Kawana et al., 2014). Upon activation, three active processes are proposed; a) generation of mitochondrial ROS from pore-forming toxins to produce protein ‘X’ hence amplifying the NLRP3 activity, b) damage to the endosome or lysosome from phagocytosis of particulate matter leading to induction of caspase-1 activity and c) potassium efflux that might be related with high extracellular calcium and ATP, although the mechanisms are unclear. The end result of these actions is NLRP3 inflammasome activity (Menu and Vince, 2011).

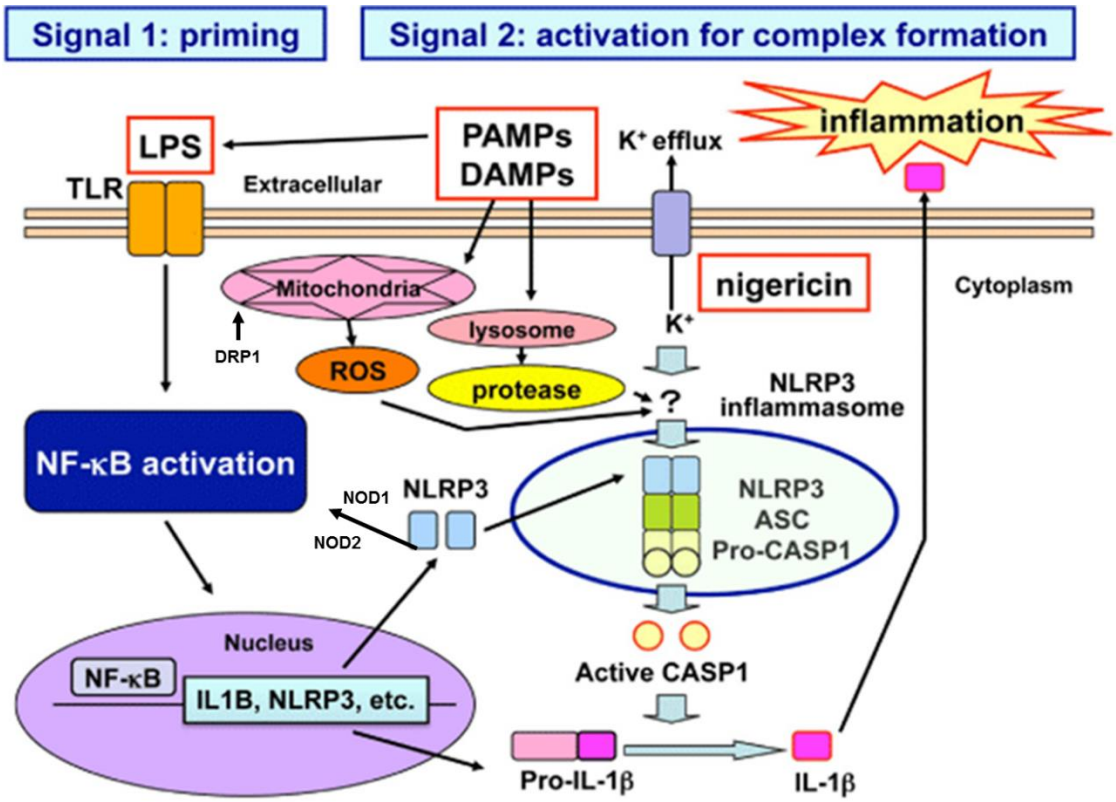

Figure 1-7 Two-step signal of NLRP3 inflammasome activation

Signal 1 or priming, enhances the NLRP3 and pro-IL-1β transcription via NF-κB activation. Signal 2 or activation, assembles the inflammasome components and causes pro-caspase-1 activation releasing active IL-1β. NOD1 and NOD2 are cytosolic proteins that trigger signal transduction via NF-κB. DRP1 induces mitochondrial fission, which leads to increased level of ROS and activated NLRP3 inflammasome (Adapted from Kawana et al., 2014).

Microbe-derived lipopolysaccharide (LPS) is a potent priming agent whereas PAMPs and DAMPs are both activation signals. Examples of PAMPs that activate the NLRP3 inflammasome include influenza A virus, *Candida albicans*, nigericin from (*Streptomyces hygroscopicus*), aerolysin (*Aeromonas hydrophila*), maitoxin (*Marina dinoflagellates*), gramidin (*Bacilus brevis*) and α-toxin (*Staphylococcus aureus*). Asbestos, uric acid crystals, silica, alum, hyaluronan, cholesterol crystals and ATP are DAMPs that activate the NLRP3 inflammasome (Petrilli et al., 2007). Reports also suggest that signals solely activating NF-κB are not sufficient to produce NLRP3 inflammasome activation; a combination of priming and activation is required (Bauernfeind et al., 2009).

The NLRP3 protein is the most widely studied of the NLR type of inflammasomes which reacts and responds to various pathogens such as bacterial, viruses and fungi. Mutation of the NLRP3 gene results in alteration of cryopyrin protein (as in **Figure 1-7**) which is associated with familial cold autoinflammatory response, neonatal onset multisystem inflammatory disease and Muckle-Well syndrome by producing increased IL-1β (Hoffman et al., 2001).

1.4 Macrophages and NLRP3 inflammasome

NLRP3 inflammasome priming and activation in macrophages is triggered by cholesterol crystallization via oxidized LDL (modified LDL) (Duewell et al., 2010). Oxidized LDL activates NF-κB via receptor complex; TLR4/6 and CD14 (Miller et al., 2003, Stewart et al., 2010). Furthermore, ROS production by macrophages and surrounding epithelial cells leads to lysosomal damage hence activates NLRP3 inflammasome (Napoli et al., 2001, Yuan et al., 1997). Duewell et al. (2010) also proved that the knockout mice with NLRP3-deficiency markedly decreased atherosclerosis via an IL-1β dependent pathway.

A cholesterol crystal is a common constituent of atherosclerotic lesions and promotes NLRP3 inflammasome activation and IL-1β secretion in macrophages by lysosomal destabilisation (Shenoy et al., 2012). The mechanism of NLRP3 activation by cholesterol crystals involves both potassium efflux and cathepsin B leakage into the cytoplasm. In atherosclerosis, the inflammasome-mediated IL-1β release promotes an inflammatory cascade and accelerates the lesion progression (Rajamaki et al., 2010) (**Figure 1-8**).

In human macrophages, NLRP3 inflammasome activation is also dependent on signalling receptor for priming. IRAK4 (interleukin-1 receptor-associated kinase 4) deficient macrophages showed caspase-1 reduction due to inability of adaptor MyD88 linked with the ligands for TLR, hence inability to cleave IL-1β and IL-18 (Bauernfeind et al., 2009). Interestingly, IL-1α secretion in human macrophages is independent from caspase-1 (Gicquel et al., 2015).

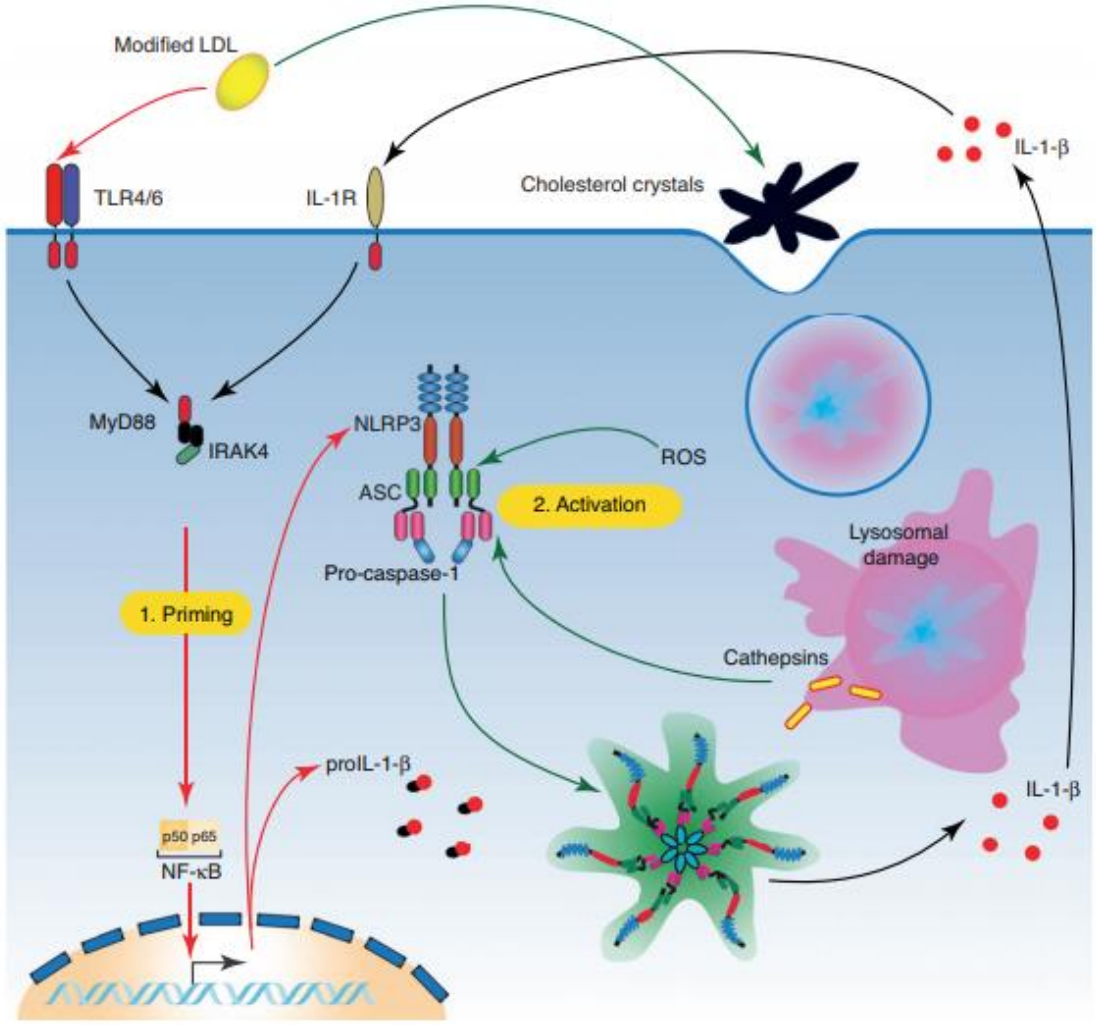

Figure 1-8 Modified LDL triggers atherosclerosis via NLRP3 inflammasome.

Modified LDL recognizes TLR4/6 and scavenger receptors of the macrophages (p50 and p65). Subsequent NF-κB activation (priming) promotes oligomerization of Signal 1 components. Macrophages also phagocytose cholesterol crystals which leads to lysosomal rupture and cathepsin release. The combination of these mechanisms triggers caspase-1 activation and cleaved active IL-1β are released (De Nardo and Latz, 2011).

1.5 Endothelial cells and NLRP3 inflamamsome

Endothelial cells play several roles in human physiology (**Figure 1-9**). Endothelial cells form a barrier that separates blood from tissue by kinase regulation (Stevens et al., 2000). Inhibition of non-muscle myosin light chain kinase enzyme initiate actomyosin interaction hence prevented G_q-linked agonists from reducing cell-cell adhesion and endothelial barrier integrity. Furthermore, endothelial cells promote vasodilation in response to acetylcholine and regulated by EDRF (endothelium-derived relaxing factor) and ECRF (endothelium-derived contracting factor) (Vanhoutte, 2003). Endothelial cells also possess anti-thrombotic properties by anti-thrombin III binding site activation and thrombomodulin expression (Sagripanti and Carpi, 2000).

Many studies about the integrity and functions of endothelial cells in inflammation*, in vivo* or *in vitro,* have been performed (Galley and Webster, 2004) **(Figure 1-9)** but inflammasome pathways are less studied (Michiels, 2003). Even though the NLRP3 inflammasome has been widely investigated in macrophages, less research has focused on its possible involvement in endothelial cells. Here the current understanding of the relationship between the NLRP3 inflammasome and endothelial cell function is described.

Both human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC) express NOD1, which is a subunit of NLRP3 inflammasome (Opitz et al., 2005) (as in **Figure 1-7**). Furthermore, low basal expression of NOD2 is potentiated with exposure to inflammatory mediators by activation of NF-kB (Opitz et al., 2006). NOD1 and NOD2 detect peptidoglycan fragments of the degradation products of bacteria, predominantly NOD1 for the gram-negative bacteria and NOD2 for the gram positive. As a consequence, these bacterial products are endocytosed into the cell cytosol or via peptide transporter (PepT1 and PepT2) (Opitz et al., 2009).

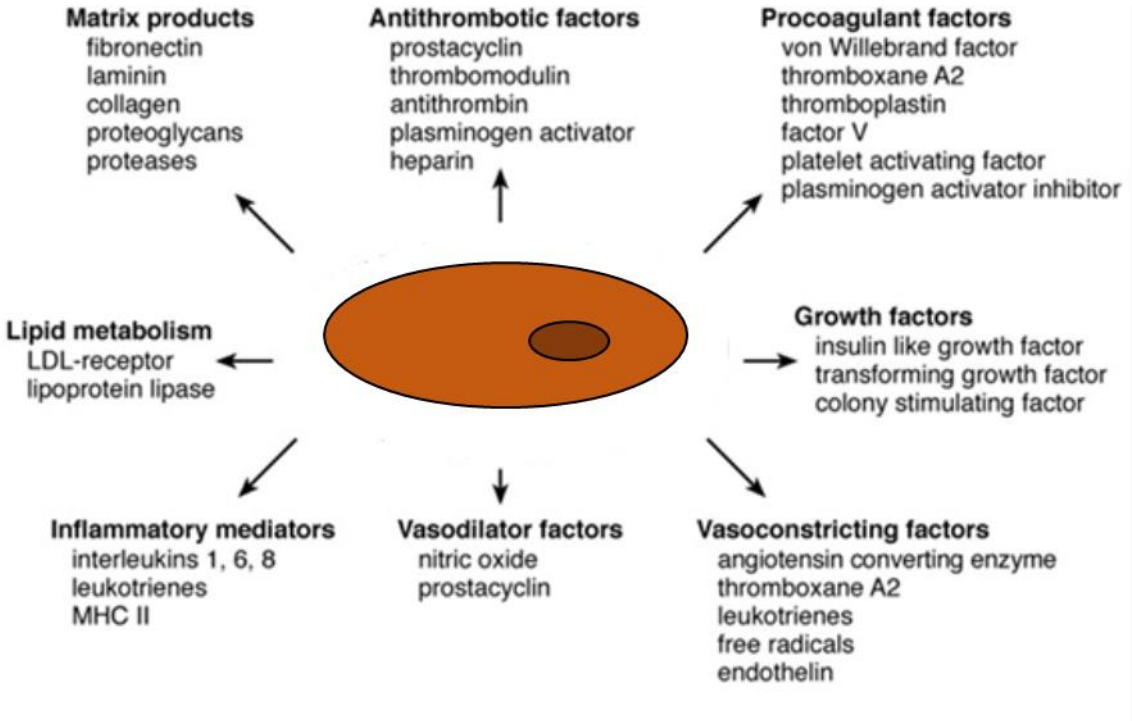

Figure 1-9 The functions of endothelial cells.

The many and varied functional roles of endothelial cells are illustrated. One of the functions of the endothelial cells is to release IL-1 (Galley and Webster, 2004).

A study in the Kawasaki disease mouse vascular endothelial cells (MVEC) model treated with *Lactobacillus casei*, which induced coronary arteritis, revealed a release of lysosomal cathepsin B protease. Cathepsin B undergone lysosomal membrane permeabilization by loss of lysosomal contents and acidity which might be responsible for proteolytically activating NLRP3 inflammasome. Furthermore, increased expression of vascular cell adhesion molecule 1 (VCAM-1) and endothelial-leukocyte adhesion were revealed by immunohistochemistry (Chen et al., 2015).

Chen et al. (2016) also demonstrated that cadmium activated NLRP3 inflammasome in HUVECs by accumulation of intracellular ROS including mitochondria ROS (mtROS). Furthermore, endothelial NLRP3 inflammasome was activated by ATP and xenobiotic pregnane X receptor (PXR) agonists. It was well observed that oxidized LDL and cholesterol crystal triggered the foam cell

formation, hence leading to atherosclerosis progression and NLRP3 activation in vascular endothelial cell (Chen et al., 2016).

There is a relationship between inflammasome signalling pathway and high mobility group box 1 protein (HMGB1) release from endothelial cells. HMGB1 Is a multifunctional protein which functions as a non-histone DNA-binding nuclear protein (Fiuza et al., 2003). In addition to IL-18 and HMGB1, fibroblast growth factor is also released under caspase-1 activation. These protein molecules are believed to release pro-inflammatory cytokines including TNF-α and pro-IL-1β via NF-κB, hence priming the inflammasome activity (Peeters et al., 2015). In lung endothelial cells, NLRP3 inflammasome is believed to be activated by endothelial NADPH oxidase through HMGB1 in the event of haemorrhagic shock or acute lung injuries. In addition, polymorphonuclear neutrophil NADPH oxidase also is required to augment the ROS production in lung endothelial cells to promote NLRP3 inflammasome production (Xiang et al., 2011).

Microparticles are generated from cells following apoptosis or during cell activation. Microparticles stimulate NF-κB and AP-1 (activator protein) pathway in synovial fibroblast, however, siRNA inhibit AP-1 signalling). A previous study of microparticles in LPS-treated monocytic cells demonstrated the activation of NLRP3 inflammasome in HUVEC via extracellular signal-regulated kinases (ERK) 1 and 2 and NF-κB, as evidenced by IL-1β production. Subsequently, the cell adhesion molecules ICAM-1, VCAM-1, and E-selectin were induced, hence promoting a pro-inflammatory phenotype (Wang et al., 2011).

Endothelial cells and inflammation play crucial roles in developing atherosclerosis **(Figure 1-10)**. The macrophage NLRP3 inflammasome can be activated by cholesterol crystals (Koenig and Khuseyinova, 2007). As a result, sub-endothelial accumulations of cholesterol, lipids, T-cells and particularly macrophage foam cells form a fatty streak which is an early lesion of atherosclerosis (Hansson et al., 2006). Deletion of the IL-1β gene in a murine model of atherosclerosis showed 30% reduction of atherosclerotic plaques (Garg, 2011).

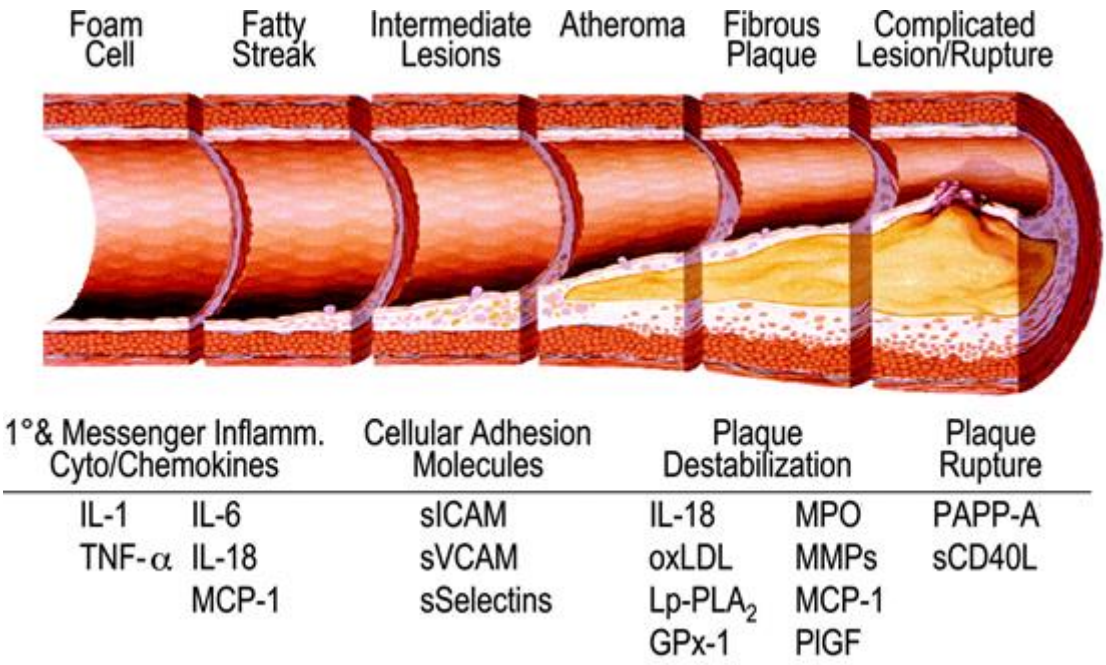

Figure 1-10 Markers of inflammation and plaque instability.

The progression of atherosclerosis from foam cell to plaque rupture. Note the involvement of IL-1β and IL-18 throughout the pathology (Koenig and Khuseyinova, 2007).

Interestingly, an on-going multinational collaborative clinical research study, introduced since 2011 is studying the effect of IL-1β inhibition; CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study) (Ridker et al., 2011). Canakinumab is a monoclonal antibody targeting IL-1β. Thousands of patients with stable post myocardial infarction and persistent elevation of CRP (C-reactive protein), a pro-inflammatory biomarker, were randomly selected for this study. Recent result showed that the magnitude of reduction in CRP was strongly related to canakinumab therapy in the absence of any change in LDL cholesterol (Ridker et al., 2018). CANTOS also demonstrated the benefits of IL-1β neutralization in patients with residual inflammatory risk post myocardial infarction (Vromman et al., 2018).

Plasma of peripheral arterial disease patients stimulated with cholesterol crystals and co-stimulated in the human aortic endothelial cells are proven to trigger the activation of endothelium intracellular signals of NLR receptors in macrophages hence activates the caspase-1. As a result, active caspase-1 cleaves pro-IL-1β

to active IL-1β, a potent pro-inflammatory cytokine resulting in atheroma formation. Even though the role of the macrophages in the onset of the atheroma plaque is essential, the vascular endothelial cell (EC) and where its dysfunction are the main onset of the development of the atherosclerosis (Aurora et al., 2009). However, this model demonstrates the higher activation of NLRP1 as compared to NLRP3 (Bleda et al., 2014). Furthermore, it is known that oxidized low-density lipoprotein (OxLDL) and oscillatory shear stress increase integrin $\alpha 5$ in lipid rafts of endothelial cells, which is involved in activation of NF-κB, hence forming the NLRP3 inflammasome which contributes to atherosclerosis (Sun et al., 2016a).

In endothelial cells, in addition to oscillatory shear stress, vascular damage and pathogen-associated molecules (such as lipopolysaccharide and cholesterol crystals) induce the activation of sterol regulatory element binding protein 2 (SREBP2) (Espenshade and Hughes, 2007). SREBP2 is a key regulator of cholesterol synthesis. Activation of SREBP2 stimulates NOX2 and NLRP3 transcription, together with the elevation of the short-lived ROS. As a result, increased ROS and the high level of NLRP3 expression lead to NLRP3 inflammasome formation. It is believed that the principal end product of NLRP3 inflammasome activation, IL-1β, promotes the production of monocyte chemoattractant protein 1 (MCP-1), VCAM-1, ICAM-1 and E-selectin, foam cell formation and monocyte recruitment, thus resulting in endothelium dysfunction and atherosclerosis (Xiao et al., 2013).

IL-18 is believed to promote atherosclerosis by IFN-γ secretion (Tenger et al., 2005). Furthermore, the combination of both cytokines (IL-18 and IL-12) induce the IFN-γ production in macrophages and smooth muscle cells of blood vessels, hence accelerating the atherosclerosis. Previous study has also described that exogenous IL-18 enhances atherosclerosis in an IFN-γ-dependent manner in the apoE KO mouse (Whitman et al., 2002).

Disturbed blood flow in narrow vascular regions especially the aortic arch and branch points is also believed to promote the expression of thioredoxin interacting protein (TXNIP) (VanderLaan et al., 2004). TXNIP is a member of the α-arrestin family, acting as inhibitor of Kruppel-like factor 2 (KLF2) expression in endothelial

cells, which is a potent anti-inflammatory transcription factor in endothelial cells. As a result, it promotes cell adhesion molecule expression as well as upstream regulation of NLRP3 protein transcription. Furthermore, there is evidence that steady blood flow promotes MEK5-ERK5 pathways, mitogen-activated protein kinase (MAPK) family of protein kinases and increased expression of KLF2 (Wang et al., 2012). Other than disturbed blood flow, shear stress or oxidized LDL particles and hyperphosphatemia also contribute to endothelial dysfunction in chronic kidney disease or uraemic patients. It is demonstrated that uraemic DAMPs causes overexpression of TLR4 and NLRP3 inflammasome components through NF-κB activation in endothelial cells (Martin-Rodriguez et al., 2015).

There are some reports that infectious agents promote NLRP3 inflammasome activation in endothelial cells. For example, cerebral malaria infection by *Plasmodium falciparum* produces erythrocyte adhesion in brain capillary endothelial cells due to excessive secretion of the inflammatory cytokine, β-hematin. β-hematin, a heme polymer hemozoin is formed after the polymerization of haemoglobin degradation product by *P. falciparum* and induced the release of IL-1β in macrophages through NLRP3 inflammasome activation (Reimer et al., 2010). On the other hand, α-hemolysin toxin from *Staphylococcus aureus* activates caspase-1 cleavage by cysteine proteinase enzyme (Craven et al., 2009). Furthermore, in endothelial cells, α-hemolysin induces platelet activating factor, which will trigger the inflammatory and thrombotic cascades (Suttorp et al., 1992).

Importantly, there is a strong relationship between vascular endothelial dysfunction and mitochondria-driven oxidative damage, specifically mtROS (Dromparis and Michelakis, 2013). Electron transport chain (ETC) Complex 1 of mitochondria is disrupted by oxidative stress. Additionally, exposure of endothelial cells to high glucose and free fatty acid, as in metabolic disease individuals, increases mtROS hence triggers atherosclerosis (Du et al., 2006). Interestingly, tobacco smoke triggers mitochondrial depolarization and endothelial cells apoptosis (Vayssier-Taussat et al., 2001).

1.6 Mitochondria and NLRP3 inflammasome

Mitochondria are best known as a major source of ATP supply for cell (Brookes et al., 2004). Classically mitochondria are depicted as rod shaped with two special membranes with different permeability. The inner membrane of mitochondria forms the cristae to increase the capacity of the mitochondrion to synthesize ATP (Paumard et al., 2002) (**Figure 1-11**). Furthermore, mitochondria are essential for signal transduction and programmed cell death (Friedman and Nunnari, 2014). Additionally, mitochondrial DNA (mtDNA) is embedded in the matrix which is essential for cells to maintain the respiratory competency and is inherited as a protein-DNA complex known as nucleoid (Chen et al., 2005). Some protein synthesis is achieved by the presence of ribosomes.

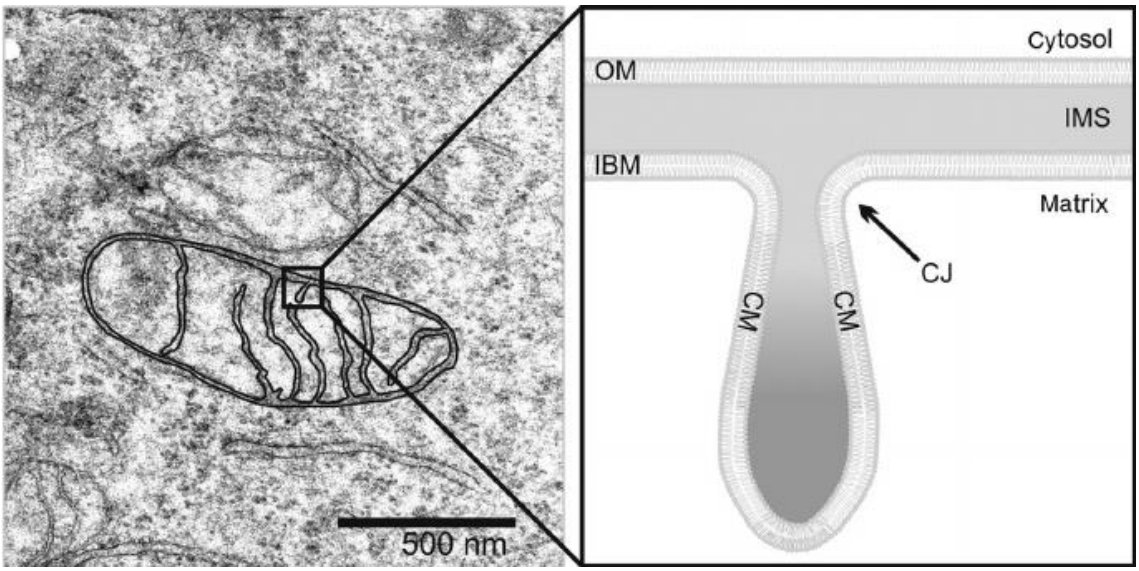

Figure 1-11 Ultrastructure of mitochondria.

Mitochondrial ultrastructure and subcompartments. Electron micrograph (left panel) represent a mitochondrial section. The schematic diagram (right panel) represents a single crista. CJ: crista junction, CM: cristae membrane, OM: outer membrane, IBM: inner boundary membrane and IMS: intermembrane space (Koob and Reichert, 2014).

A link between mitochondria and innate immune responses has been developed (Seth et al., 2005). PAMPs, environmental exposures and aging are considered the major factors leading to mitochondrial stress. This mitochondrial dysfunction manifests as accumulation of unfolded proteins, disrupted membrane action potential, impaired oxidative phosphorylation and increased ROS production. Furthermore, these effects disrupt mitochondrial membrane integrity thus releasing DAMP, mtROS or mtDNA (mitochondrial DNA) (**Figure 1-12**).

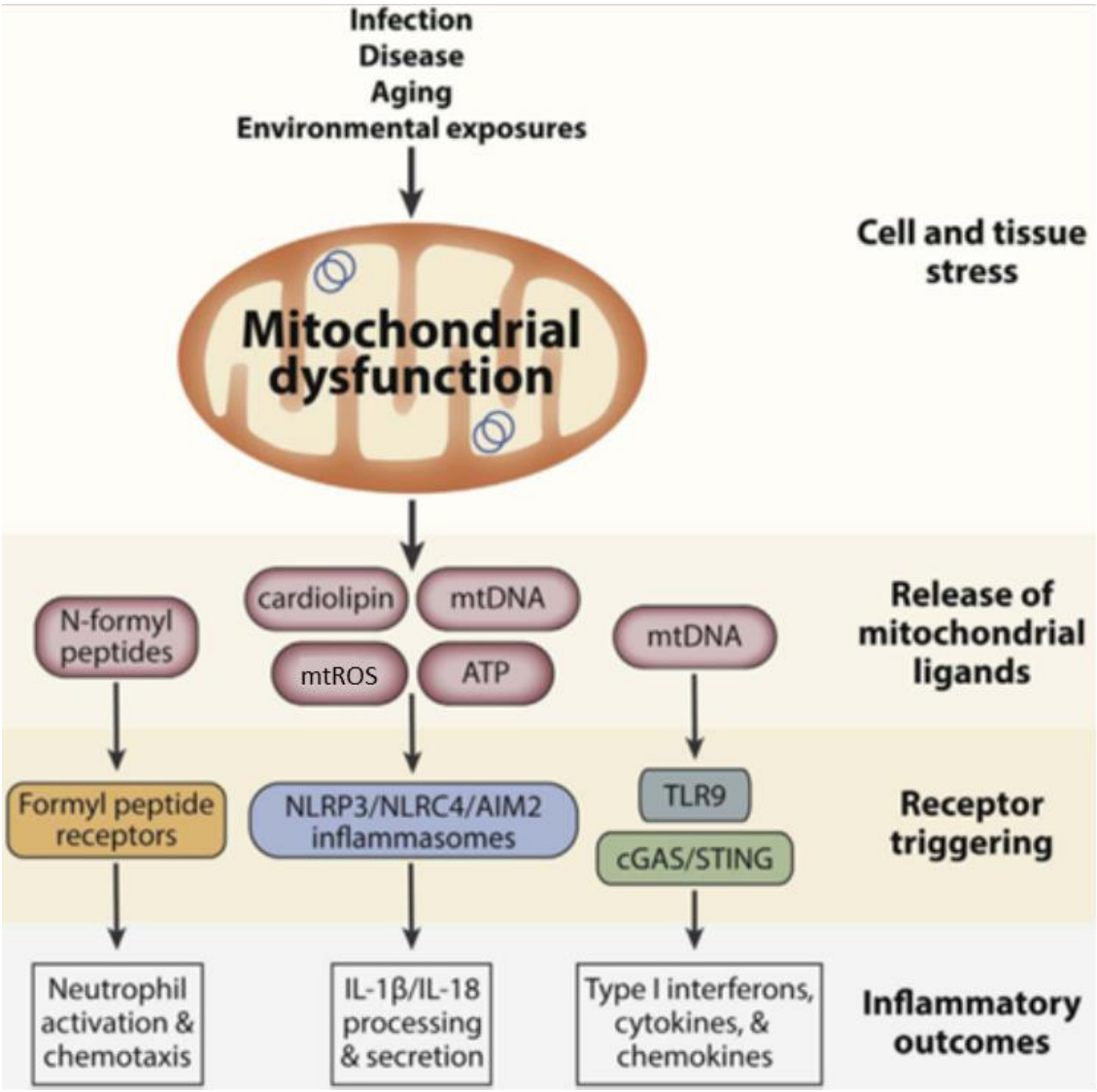

Figure 1-12 Mitochondrial stress as a trigger of innate immune responses.

Cell and tissue stress can directly or indirectly cause mitochondria dysfunction. This process effectively disrupts mitochondrial membrane integrity, leading to the release of mitochondrial ligands. These molecules then activate PRR of the innate immune system, hence triggering inflammatory responses (West, 2017).

Notably, NLRP3 inflammasomes and mitochondrial dysfunction interact in inflammatory diseases such as gout, autoimmune disease and atherosclerosis. Although NLRP3 inflammasomes may respond to many cellular stressors, further study might be needed to delineate exact mechanisms of activation. Furthermore, a number of stress stimuli such as hypoxia and increased metabolic rate may increase mtROS production which leads to NLRP3 inflammasome activation (Brookes et al., 2004).

Leakage of electrons from ETC (electron transport chain) react with the oxygen to produce intracellular free radical superoxide (Indo et al., 2007). Interestingly, rotenone activates inflammasome Signal 1 in the presence of ATP via disruption of mitochondrial electron transfer (Won et al., 2015).

Multiple studies concluded a strong relationship between ROS production and NLRP3 inflammasome activation Signal 2 by NOX2 which might be due to mitochondria Complex I disturbances (Franchi et al., 2014, Iyer et al., 2013, Juliana et al., 2012, Nakahira et al., 2011, Shimada et al., 2012a, Zhong et al., 2013). Additionally, mitochondria and NADPH oxidase (NOXs) lead to cellular ROS production such as superoxide and hydrogen peroxide. Increased cytosolic Ca^{2+} level also leads to NLRP3 inflammasome activation in addition to mitochondrial damage and mtROS production (Triantafilou et al., 2013). However, proteins involved in the response to oxidative stress; TXNIP and Nrf2 (nuclear factor-like 2) contributed to NLRP3 inflammasome activation in a caspase-1 independent pathway (Dunn et al., 2015).

Mitophagy or mitochondria-specific autophagy is a programmed process to eliminate damaged or unwanted mitochondria and functions to preserve the mitochondria equilibrium (Youle and Narendra, 2011) (**Figure 1-13**). Damaged mitochondrial undergo Parkin-dependent ubiquitin conjugation and are specifically recognised by p62, a multifunctional signalling scaffold and adaptor in macrophages. Subsequently, the triggered p62 receptor is translocated to damaged mitochondria for mitophagy stimulation (Zhong et al., 2016).

Additionally, study in a sepsis model demonstrated SESN2 (sestrin 2), a stress-inducible protein, suppresses NLRP3 inflammasome activation by clearance of damaged mitochondria through mitophagy (Kim et al., 2016a). Furthermore, dysfunctional mitophagy allows unhindered NLRP3 inflammasome activation via mtROS (Nakahira et al., 2011).

Besides mtROS, mitochondrial DAMPs (mtDAMPs) following *Chlamydia* and *Salmonella* infection are able to activate NLRP3 inflammasomes by releasing oxidized mtDNA. Bacterial cardiolipin or phospholipid attached to the inner mitochondrial membrane, activate ROS-dependent pathways of mitochondrial damage (mtDAMPs) as an NLRP3 activator.Some viruses such as rabies are able to cleave the mitochondria thus activating the outer mitochondrial membrane GTPase dynamin-related protein 1 (Drp1) or mitofusin 1 and 2. Drp1 is necessary for NLRP3-mediated inflammation (Ichinohe et al., 2013) (as in Figure 1-7).

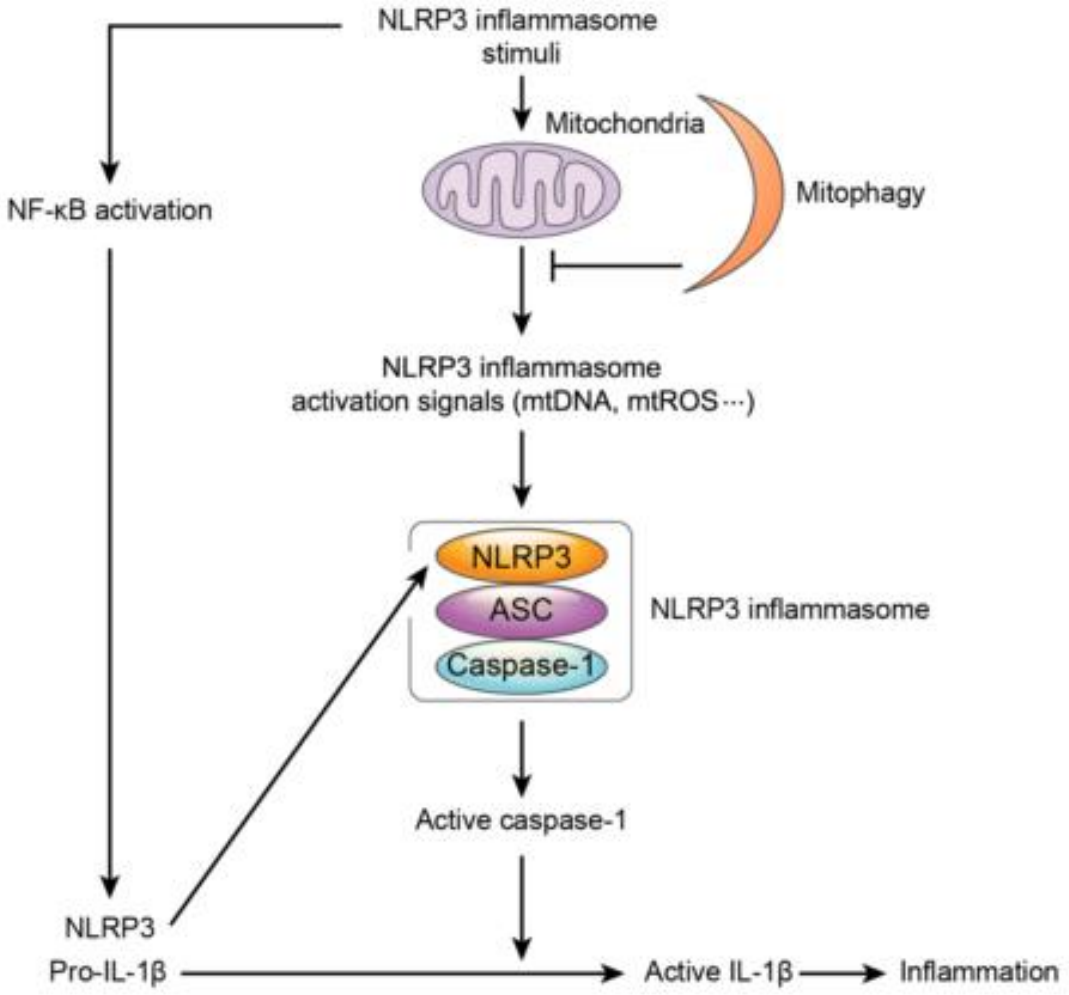

Figure 1-13 Mitophagy downregulates NLRP3 inflammasome activation.

Mitochondrial damage either by external stimulation or NLRP3 inflammasome activation signals trigger the mitophagy process. Subsequently, mitophagy downregulates active caspase-1 and active IL-1β production by blocking NLRP3 inflammasome cascades (Kim et al., 2016b).

1.7 Hydrogen sulfide and NLRP3 inflamamsome

1.7.1 Endogenous hydrogen sulfide synthesis

Hydrogen sulfide (H_2S) has been studied for more than 20 years in mammalian cells, basically as a toxic gas in higher concentration (Reiffenstein et al., 1992). This molecule belongs to the gasotransmitter family of signalling molecules that includes nitric oxide and carbon monoxide. H_2S has physiological functions such as smooth muscle relaxation, insulin regulation, neuronal transmission modulation as well as being a potent signalling molecule and cytoprotectant in lower concentrations (Feelisch and Olson, 2013). Furthermore, it has been identified as an oxygen sensor or transducer under stress conditions especially in hypoxia (Olson et al., 2006).

Previously, H_2S has been extensively studied in the nervous and vascular systems (Yang et al., 2008). However, it is becoming of interest in many organs such as liver, kidney, spleen, small intestine, pancreas and adipose tissue (Belardinelli et al., 2001). H_2S production is controlled by a series of enzymatic and non-enzymatic reactions which might give certain clinical signs whenever the pathway is disturbed such as the development of cardiovascular disability in patients with cystathionine β-synthase (CBS) genetic deletion (Vandiver and Snyder, 2012).

Homocysteine and cysteine, sulfur-containing amino acids, are metabolized and produce H_2S (Li et al., 2011). As in **Figure 1-14**, generally, three main enzymes are involved in H_2S production or co-called desulfhydration of L-cysteine; cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST) coupled with cysteine aminotransferase (CAT). CBS and CSE are found in cytosol, whereas CAT is present in both cytosol and mitochondria, depending on the specific organ and species (Chiku et al., 2009). Furthermore, a more recent study demonstrated the novel production of H_2S by D-cysteine which is primarily present in cerebellum and kidney (Shibuya et al., 2013).

H_2S which has been produced in cytosol is able to directly disperse into the mitochondria before being metabolised, hence generating mitochondrial ATP production in an oxygen-dependent fashion (Fu et al., 2012). 3MST acts as an endogenous bioenergetic factor that donates electrons to sulfide quinone oxidoreductase (SQR) hence coupling the mitochondrial electron transport to aerobic ATP generation (Módis et al., 2013). Furthermore, H_2S has been shown to regulate blood vessel dynamics by triggering K_{ATP} channel-mediated vasorelaxation *via* acting as a K_{ATP} channels ligand (Zhao et al., 2001).

In cytosol, the steps of H_2S production begin with two amino acids; serine and methionine. Methionine is converted by S-adenosylmethionine, a methylation reaction to form homocysteine (Kalhan and Hanson, 2012). CBS is involved in the production of H_2S by trans-sulfuration of homocysteine to cystathionine. Subsequently, CSE metabolises cystathionine to form cysteine and α-ketobutyrate. Finally, H_2S is produced by elimination (CSE) or by β-replacement (CBS) (**Figure 1-14**).

In mitochondria, the mercaptopyruvate pathway is one of the major enzymatic pathways of H_2S production (**Figure 1-14**). Cysteine is catalysed by CAT to form 3-mercaptopyruvate (3MP) and subsequently directly producing H_2S by 3MST, an anti-oxidant protein, hence maintaining the cellular redox state (Nagahara and Sawada, 2006). Additionally, this pathway also is regulated by the level of intracellular Ca^{2+} (Mikami and Kimura, 2012). Even though the cysteine level is higher in mitochondria, the level of CBS and CSE are relatively lower as compared to the level in cytosol.*In vivo* , in neurons and astrocytes of mice, H_2S is stored inside the cells as bound sulfane sulfur and will be released under reducing conditions when the pH is higher than 8.4 (Ishigami et al., 2009).

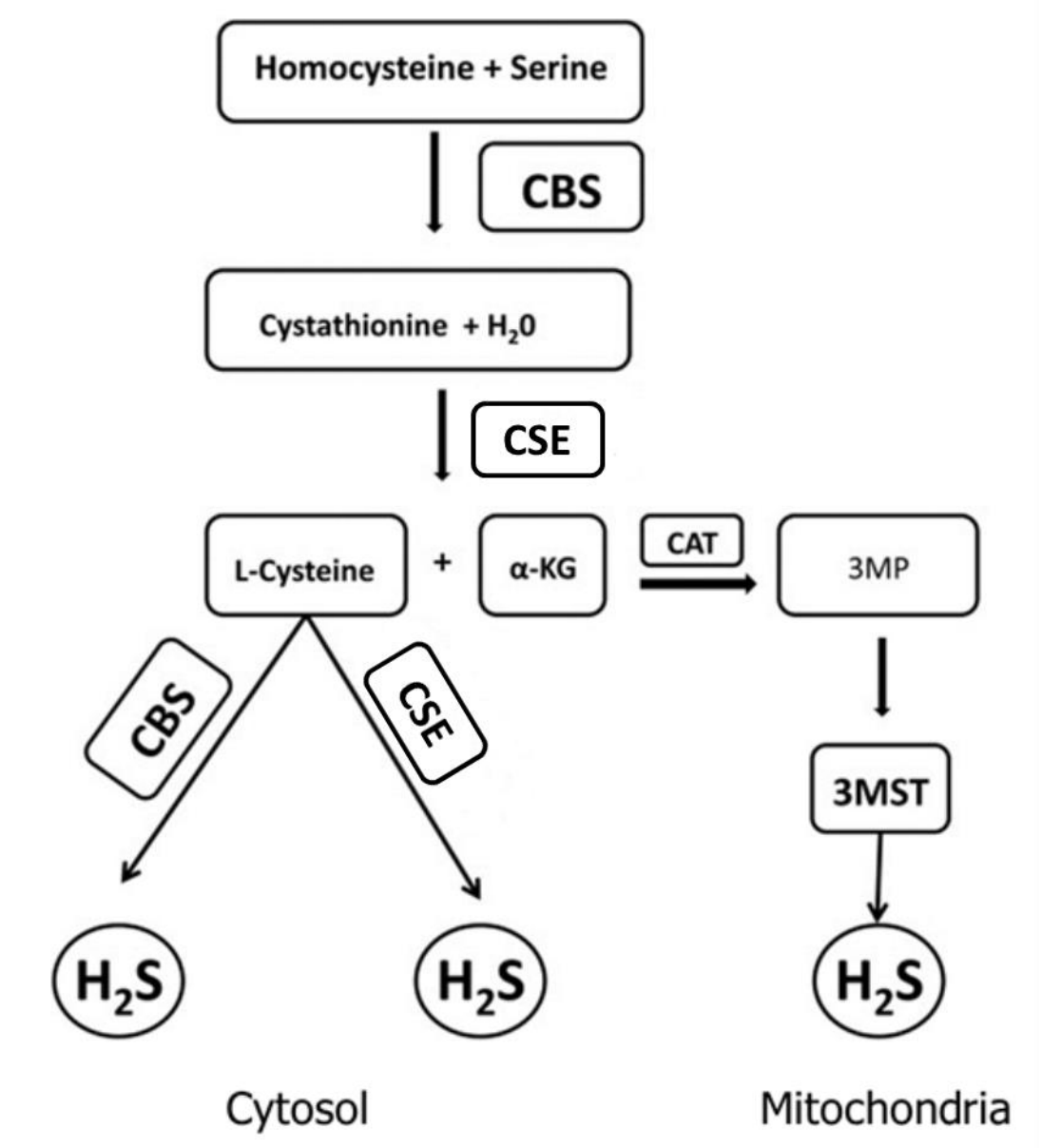

Figure 1-14 Enzymatic synthesis of hydrogen sulfide

There are three enzymatic pathways involved in the synthesis of H_2S in mammalian systems. CBS produces H_2S through a reaction involving the generation of cystathionine from homocysteine and L-cysteine from cystathione. CSE produces H_2S through a reaction involving the generation of L-cysteine from cystathionine. In mitochondria, 3MST produces H_2S through a reaction involving the generation of 3MP from α-ketoglutarate (α-KG) by CAT. Adapted from (Moody and Calvert, 2011).

Previous studies demonstrated the effect of CSE inhibitors; D,L-propargylglycine (PAG) and β-cyano-L-alanine (BCA). However, they are not effective in inhibiting brain H_2S production (Bailey and Pluth, 2013). On the other hand, aminooxyacetic acid (AOAA), is commonly used as a potent CBS inhibitor but there is no selective CBS inhibitor to date (Asimakopoulou et al., 2013). Surprisingly, there is no widely available specific inhibitor for 3MST even though a novel study using a fluorescence probe had described the discovery of small molecules inhibiting 3MST (Shimamoto et al., 2014).

Even though there is no specific biomarker of H_2S catabolism, the rate and extent of H_2S chemical and biochemical catabolism varies from one organ to another. H_2S is metabolized in mitochondria and is removed quickly from the cellular environment especially under aerobic conditions (Vitvitsky et al., 2012). Three main enzymes involved in H_2S metabolism are CAT, cysteine dioxygenase (CDO) and cysteine lyase (CL) (Yin et al., 2016).

1.7.2 Role of hydrogen sulfide in human biology

Even though numerous early studies mentioned the adverse effects of H_2S, it was then demonstrated to be an endogenous physiological neuromodulator as evidenced by high CBS and H_2S levels in brain (Abe and Kimura, 1996). Furthermore, N-methyl-D-aspartate receptor (NMDA) is believed to be stimulated by an optimum level of endogenous H_2S in the hippocampus hence triggering the messenger in hippocampal long-term potential (LTP), as a synaptic model for memory which might be associated with learning ability (Kimura, 2011).

H_2S also aggravates Ca^{2+} influx in astrocytes by electrical stimulation of nearby neurons hence regulating NMDA receptors which are activated by glutamate or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Eto et al., 2002). Thus, the adverse effect of excess glutamate as well as H_2O_2 toxicity might be protected against by H_2S (Nagai et al., 2004).

High levels of CSE mRNA in smooth muscle cells increase H_2S production which leads to K^+ efflux in a non-ATP-associated manner, hence promoting smooth muscle proliferation by membrane potential hyperpolarization (Dominy and

Stipanuk, 2004). Physiologically, extensive smooth muscle proliferation is terminated by H_2S by triggering the cyclin-dependent kinase inhibitor p21 and ERK phosphorylation (Yang et al., 2004). Interestingly, in smooth muscle relaxation, NO activity is also amplified by CSE (Oh et al., 2006a).

H_2S plays an extensive role in cardiovascular system physiology. In blood vessels, H_2S demonstrates vasorelaxation in a concentration-dependent manner by the effect of specific catalytic activity of CSE. H_2S also induces angiogenesis by the Akt signalling pathway (Cai et al., 2007). Furthermore, a lower level of H_2S has a protective effect as an antioxidant towards homocysteine-induced toxicity in blood vessels (Kamat et al., 2013). An anti-atherosclerotic effect of H_2S also has been demonstrated in hypochlorite-induced oxidative LDL modification (Laggner et al., 2007).

Oxygen level also has a major influence on H_2S-related blood vessel regulation. Hypoxia causes rapid vasodilatation whereas hyperoxia induces vasocontriction (Koenitzer et al., 2007). Furthermore, different O_2 concentrations may affect the release of NO by H_2S from *S*-nitroglutathione. Lysates of endothelial cells produce H_2S from 3MP and cysteine in the presence of a-ketoglutarate (Kimura, 2011).

The cardioprotective activity of H_2S has been studied previously by many researchers. Ischaemic preconditioning with H_2S is believed to have a cardioprotective role via K^+ channel opening which is produced by extracellular signal–regulated kinases (ERK) 1/2 activation (Hu et al., 2008). Additionally, in electron microscopy findings , H_2S preserved mitochondrial ultrastructure by decreased haemorrhage, necrosis and leukocyte transmigration, reduced cardiomyocyte apoptosis and reduced myocardial inflammation (Elrod et al., 2007). H_2S also has anti-platelet effects, disrupting aggregation in response to ADP and collagen (Zagli et al., 2007).

Even though much research has been performed in investigating the inflammatory effect of H_2S, both pro- and anti-inflammatory pathways of H_2S are complex and varied (Whiteman and Winyard, 2011). This comprehensive review

demonstrated that the duration of inflammation, the timing of H_2S administration and the concentration of H_2S plays a major role in inflammatory effects of H_2S.

A few clinical disorders are known to be related with genetic alterations of hydrogen sulfide metabolism. Human mercaptolactate-cysteine disulfiduria (MCDU) is manifest by mental retardation and excessive excretion of 3-mercaptolactate-cysteine disulphide in urine and is caused by lack of 3MST enzyme (Nagahara et al., 2013). Furthermore, patients with CBS or CSE defects presented with homocystinuria and cystathioinuria, respectively (Wijekoon et al., 2006). Inherited sulphite oxidase deficiency is a rare H_2S metabolism disorder where the infants present with seizures and alterations of muscle tone. Urinary sulfite, thiosulfate and S-sulfocysteine levels were elevated in all cases (Tan et al., 2005). In Down's syndrome, individuals are born with normal intelligence which starts to decline within the first year of life. Since the gene for CBS is located on chromosome 21, CBS hyperactivity was detected in fibroblasts of Down's syndrome patients with phenylketonuria (Kamoun, 2001).

1.7.3 Hydrogen sulfide chemical donors

H_2S donors are compounds that degrade to release H_2S, and which have their own profiles, by-products and potential therapeutic applications. They are also applicable to further evaluation of the role of H_2S in biological settings. Due to limited endogenous H_2S production, augmentation with exogenous H_2S is currently of great interest. Each of the exogenous H_2S donors manifests different structure, bioactivity and signalling effects (Wang, 2002).

Generally, the classification of H_2S donors varies depends on the research interest. However, four main classifications have been described recently; hydrolysis-triggered H_2S donor, thiol-triggered H_2S donor, light and enzyme-triggered H_2S donor and dual carbonyl sulfide, COS/H_2S donor systems. GYY4137, which is a hydrolysis-triggered H_2S donor, is the most frequently used due to ease of handling and commercial availability (**Figure 1-15**) (Powell et al., 2018).

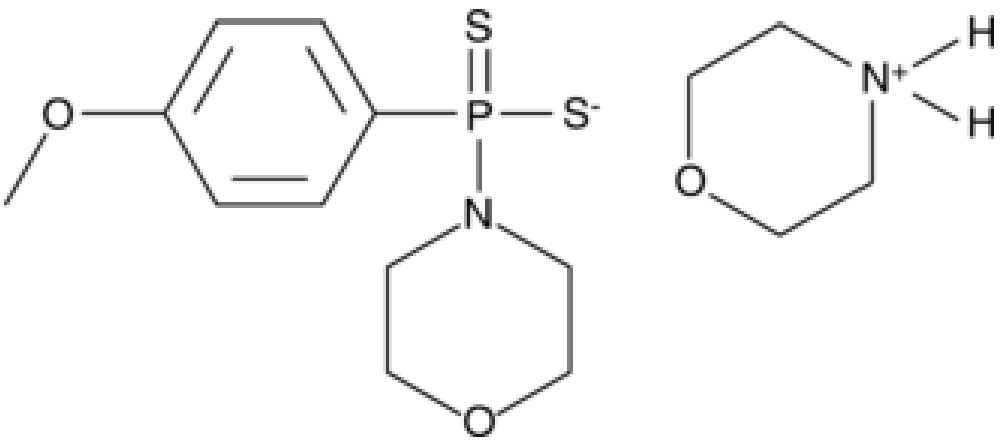

Figure 1-15 Chemical structure of GYY4137morpholine salt.

Schematic diagram shows a GYY4137 that is water-soluble and acts as a slow-releasing hydrogen sulfide donor.

Other than GYY4137, there are a few other exogenous compounds which have been classified as hydrolysis-triggered H_2S donors; sulfide salts, Lawesson's reagent (LR), phosphorodithioates and dithiolthiones (Park et al., 2013). Sulfide salts such as sodium hydrosulfide (NaHS) and sodium sulfide (Na_2S) have been used as a therapeutic measure of potential exogenous H_2S delivery, as well as potent anti-oxidants to inhibit lipid peroxidation, protein oxidation and cytoxicity (Whiteman et al., 2005). Other than anti-inflammatory action, sulfide salts are believed to have cardioprotective effects and to lead to blood sugar decrease (Wang et al., 2015). Adversely, this type of "fast releasing" or instantaneous generation of H_2S in aqueous solutions has been previously reported to have pro-inflammatory activity in liver and lungs by increased myeloperoxidase (MPO) activity hence making it a doubtful tool to study H_2S effects in physiology or pathological conditions (Li et al., 2005).

Other than sulfide salts, LR is considered a very slow release type of exogenous H_2S and it is commercially available. It was first developed in 1978 as it was used in organic synthesis especially for carbonyl group conversion to thiocarbonyls

(Scheibye et al., 1978). Even though LR is hydrophobic, it proved to have therapeutic effects such as anti-cancer, vasodilation and anti-inflammation (Zhao et al., 2014). LR prevents neutrophil infiltration, reduces oxidative stress and decreases secondary damage by inflammation in alendronate (ALD)-induced gastric damage in rats (Nicolau et al., 2013).

GYY4137 (morpholin-4-ium 4 methoxyphenyl (morpholino) phosphinodithioate), which is a water-soluble compound and releases H_2S 10 times more slowly compared to sodium hydrosulfide. GYY4137 has been discovered more than 50 years ago in the rubber industries but with a paucity of information mentioning about its biochemistry properties (Martelli et al., 2012). This type of exogenous H_2S is commonly used for research purposes especially in the cardiovascular area due to its ease of handling. Furthermore, the vasodilation effect of controlling and sustaining blood pressure by opening vascular smooth muscle K_{ATP} channels, mimicking endogenous H_2S, avoids the unwanted inotropic effect of myocardial contractility (Geng et al., 2004).

Additionally, GYY4137 has been proved to target and kill a few types of cancer cells *in vitro* without making harm to normal cells, exhibiting anti-cancer properties and cell cycle arrest for a few days in a concentration-dependent manner (Lee et al., 2011). As a residue of GYY4137 crystallization, a dichloromethane complex may be present which could mimic CO effects (Alexander et al., 2015). Investigation of GYY4137 will be further discussed in Chapter 5.

Phosphorodithioates are one of the hydrolysis-triggered H_2S donors which were developed upon hydrolysis of the core structure of GYY4137 in aqueous buffer (Park and Xian, 2015). This donor possesses slow and sustainable H_2S release and showed anti-inflammatory and anti-oxidant effects (Lee et al., 2011). Furthermore, B16BL6 cancer cell proliferation also is inhibited by phosphorodithioates (Park et al., 2013). A recent study demonstrated the production of phosphonamidothiates, by-products of GYY4137 intramolecular cyclization, which showed slow, significant and pH-controlled H_2S release. Phosphonamidothiates have been shown to protect cardiomyocytes during reperfusion injury (Kang et al., 2016).

The last family of hydrolysis-triggered H_2S donors, 1,2-dithiole-3-thiones (DTTs), formed by the reaction of sulfur and anethole, are easily synthesized and conjugated to other drugs such as non-steroidal anti-inflammatory drugs, NSAIDs (DTT-NSAIDs) (Xu et al., 2016). Pharmaceutically, this H_2S-NO releasing hybrid has anti-inflammatory properties (Kodela et al., 2012). Additionally, DTTs have anti-angiogenic and anti-cancer properties (Perrino et al., 2008).

Interestingly, in 2014, a novel compound was synthesized, AP39, which targets the mitochondria via its lipophilic triphenylphosphonium (TPP^+) bromide tail (Le Trionnaire et al., 2014, Szczesny et al., 2014) (**Figure 1-16**).The research on AP39 is still ongoing but it has been shown to inhibit apoptosis via mitochondria and preserves mitochondria respiration and ultrastructure (Le Trionnaire et al., 2014). AP39 is believed to stimulate the mitochondrial electron transport chain especially at complex IV by acting as an electron donor, hence inhibits cytochrome *c* oxidase as well as suppressing apoptosis-inducing factor thus preventing mitochondrial cell death (Szczesny et al., 2014).

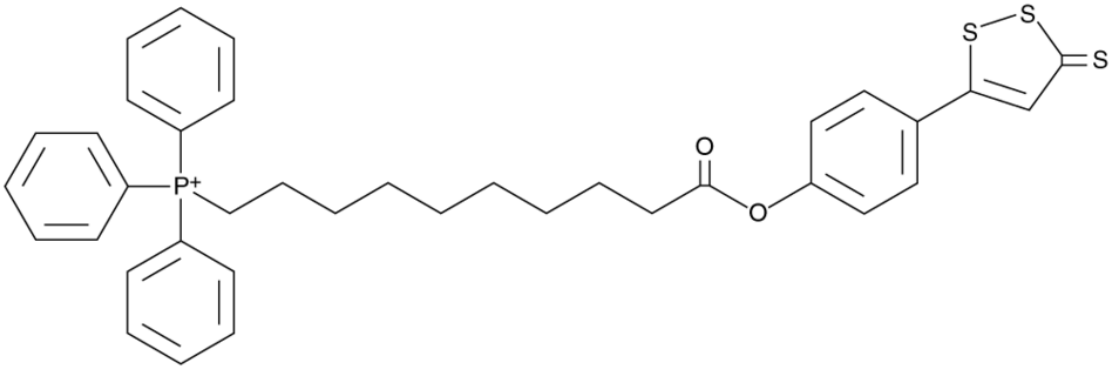

Figure 1-16 Chemical structure of AP39.

Schematic diagram shows a mitochondria-targeted hydrogen sulfide donor which consists of a mitochondria-targeting motif, triphenylphosphonium (TPP+).

1.8 Hypothesis and aims

Previous unpublished data from this group demonstrated that high glucose levels, mimicking hyperglycaemia in man, induced premature ageing of human endothelial cells in culture by causing rapid telomere attrition in a process driven by mitochondria, in particular through mitochondrial oxidant production. Mitochondria are important determinants of endothelial cell function, and hence maintain endothelial homeostasis. However, this thesis now tests the hypothesis that mitochondrial dysfunction via ROS accelerates pro-inflammatory changes via activation of the NLRP3 inflammasome. Moreover, it also tests the hypothesis that H_2S generation in mitochondria attenuates NLRP3 inflammasome activation in cells, and is therefore anti-inflammatory.

The first results chapter (Chapter 3) describes the investigation of production of the pro-inflammatory cytokines; IL-1β and IL-18 by THP-1 cells and human endothelial cells (EA.hy926 and HUVEC). Thus, the aim was to demonstrate NLRP3 inflammasome Signal 1 (Priming) and Signal 2 activation pathways in THP-1 cells as a positive control and also to investigate possible NLRP3 inflammasome Signal 1 and Signal 2 pathways in endothelial cells (HUVEC and EA.hy926 cell).

Chapter 4 focuses on mitochondrial dysfunction induced by certain intracellular superoxide generators. Thus, the aim of this chapter was to investigate the effects of mitoparaquat (mitoPQ) and paraquat (PQ) on inflammasome Signal 1 and Signal 2 in differentiated professional macrophages, THP-1 cells, before similar pathways were investigated in endothelial cells. Other pathways of NLRP3 inflammasome were investigated also in this chapter.

Subsequently, Chapter 5 concentrates on the effect of H_2S generation in THP-1 cells and whether H_2S is able to change NLRP3 inflammasome activation.

Chapter 2 Materials and methods

2.1 Materials

2.1.1 Cell lines

THP-1 cells, EA.hy926 cells and HeLa cells were purchased from American Type Culture Collection (ATCC), Virginia, United States of America.

Human umbilical vein endothelial cells (HUVEC) were purchased from Cellworks, a division of Caltag Medysystems Company, Buckingham, United Kingdom.

2.1.2 Cell culture consumables

Corning Costar Stripette 25ml, 10ml and 5ml serological pipettes, Greiner Cellstar T75 flask, Corning Centristar 10ml, 50ml centrifuge tube and Corning Pipette tips were obtained from Sigma Aldrich, Dorset, United Kingdom. Integra pipetboy was purchased from Integra-Biosciences, Zizers, Switzerland. Gilson pipette PIPETMAN was purchased from Gilson Scientific Ltd., Luton, United Kingdom. Microtubes 0.5ml and 1.5ml were purchased from Sarstedt, Leicester, United Kingdom.

2.1.3 Cell culture media

2.1.3.1 Basal media

THP-1 RPMI-1640 growth medium (500ml) was purchased from Sigma-Aldrich, Dorset, United Kingdom. Gibco Dulbecco's modified eagle medium (DMEM) 1X 500ml containing 4.5g/L glucose, L-glutamine and pyruvate was obtained from Thermo Fisher Scientific, Rugby, United Kingdom. Human large vessel endothelial cell basal medium (500ml) was purchased from Cellworks, a division of Caltag Medysystems Company, Buckingham, United Kingdom.

2.1.3.2 Cell media additives and supplements

Heat inactivated, fetal bovine serum (500ml) was obtained from Gibco, Thermo Fischer Scientific. Trypan blue solution (0.4%) was purchased from Sigma-Aldrich. Trypsin (5g/L) and EDTA (2g/L) solution (100ml) was purchased from Lonza Group, Slough, United Kingdom. L-glutamine, Corning (200mM) was obtained from VWR International, Lutterworth, United Kingdom. Phosphate buffered saline (PBS) Dulbecco A tablets were purchased from Oxoid, Fischer Scientific. Growth supplement (10ml) and antibiotic supplement (Amphotericin B/ Gentamicin) 1000X concentrate (0.5 ml) were obtained from Cellworks, a division of Caltag Medysystems Company.

2.1.3.3 Cell treatment reagents

Phorbol 12-myristate 13-acetate (PMA), (P1585) and lipopolysaccharide from Escherichia coli (10mg L2630), etoposide (25uM E1383) and MitoRed (200nM 5271) were purchased from Sigma-Aldrich. BzATP triethylammonium salt (1mg, sc-203862) was ordered from Santa Cruz Biotechnology Inc. Paraquat dichloride x-hydrate pestanal, (36541 1µM and 5µM) was from Sigma-Aldrich. Mitoparaquat, (18808 1µM and 5µM) from Cambridge Bioscience Ltd. Mitoparaquat (control) (1µM and 5µM) was received directly as a kind gift from Dr Mike Murphy from University of Cambridge. Mitoparaquat (control) is targeted to the mitochondria but does not generate superoxide anion. GYY4137 (sc-224013 100µM and 200uM) was from Santa Cruz Biotechnology, Inc. AP39 (17100-1 mg-CAY) was obtained from Cambridge Bioscience Ltd.

2.1.4 Protein extraction

Tris-base (50mM), sodium chloride (150mM), sodium deoxycholate (0.5%), hydrochloric acid (1M) and phosphatase inhibitor were purchased from Thermo Fischer Scientific. Sodium dodecyl sulphate (SDS) (0.1%), Triton-X-100 (1%) and sterile PBS was from Sigma Aldrich.

2.1.5 Protein Assay

Detergent compatible, (DC) protein assay kit was purchased from Bio-Rad, Hemel Hempstead, United Kingdom, containing alkaline copper tartrate solution for colorimetric assays (reagent A), dilute Folin reagent for colorimetric assays (reagent B) and surfactant solution for colorimetric assays (reagent S). 96 well sterile cell culture plates, flat bottom with lid were obtained from Greiner Cellstar, Sigma-Aldrich. Pierce™ Bovine Serum Albumin Standard Ampules, (23209 2mg/mL) was purchased from Thermo Fisher Scientific.

Equipment	Source
Centrifuge 5415 R	DJB Labcare Ltd., Buckinghamshire, UK
Fisons WhirliMixer	Fisher Scientific, Leicestershire, UK
NOVOstar	BMG Labtech, Bucks, UK

Table 2-1 Equipment required for protein extraction and DC protein assay.

2.1.6 Antibodies

Anti-NLRP3 antibody, (12352203 1µg/ml), rabbit polyclonal and rabbit anti-mouse (ab6728 1:10000) were from Abcam Ltd. Rabbit polyclonal to IL-18, (ABCAAB68435 1µg/ml) was from VWR International Ltd. Human IL-1β antibody, (MAB601 1µg/ml) was from R&D Systems. Caspase-1 antibody, (2225T 1:1000) was from New England Biolab (UK) Ltd. Caspase 5 antibody (cleaved Asp121) (GTX86923 rabbit polyclonal 1:500) was from Insight Biotechnology Ltd. Anti-Asc, pAb, (AL177 1:1000) was from Caltag Medsystem Ltd. HMGB1 antibody, (mab16901 1µg/ml) and goat anti-rat IgG HRP Affinity Purified (HAF005 1:1000) were from R&D Systems. Monoclonal anti-β-Actin antibody, (A5441 1:2000) was from Sigma-Aldrich Company Ltd. Goat anti-rabbit IgG-HRP (sc-2030 1:10000) was obtained from Santa Cruz Biotechnology, Inc.

2.1.7 Western blot

Tris-base, SDS, ammonium persulfate (APS), N,N,N',N-Tetramethylethylenediamine (TEMED), glycine, Tween-20, dithiothreritol, bromophenol blue, glycerol, Ponceau S staining, sodium chloride, β-mercaptoethanol and isopropanolol were purchased from Sigma-Aldrich. Hydrochloric acid, methanol, sodium hydroxide and acetic acid were obtained from Thermo Fisher Scientific. Acrylamide 30%/ Bis solution 29:1 was purchased from Bio-Rad. Enhanced chemiluminiscence reagent was obtained from Amersham Pharmacia Biotech Inc., New Jersey, United States of America. Marvel semi-skimmed milk powder was purchased from a local supermarket. Page Ruler prestained protein ladder, (26616 5µl) was obtained from Thermo Fisher Scientific.

	Resolving gel 12%	Stacking gel (4%)
dH_20 water	2.1ml	3.675ml
IM Tris – HCl	3.75ml, pH 8.8	0.625ml, pH 6.8
30% Acylamide / Bis solution 29:1	4ml	0.665ml
10% SDS	100µl	50µl
10% APS	50µl	25µl
TEMED	5µl	5µl

Table 2-2 Gel preparation of resolving and stacking gel for SDS-PAGE.

Equipment	Source
Techne Dri-block heater	Bibby Scientific Limited, Staffordshire, UK
Hybridisation oven and shaker	Stuart Scientific, Essex, UK
Weighing scale	Sartorius UK Ltd., Surrey, UK
Denley Spiramax 10 roller mixer	Denley, Sussex, UK
KCH-Vibrax-VXR shaker	Thermo-Scientific, Rugby, UK
Image Quant TL 7.0	GE Healthcare Life Sciences, Buckinghamshire, UK
Power pack, Western blot gel casting, electrophoresis and protein transfer equipment	Bio Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK

Table 2-3 The equipment required for Western blotting.

2.1.8 ELISA

Equipment	Source
MAGPIX® System	Millipore (UK) Limited, Watford, Hertfordshire, UK
Human Premixed Multi-Analyte Kit (IL-1β and IL-18)	R&D Systems, Abingdon, UK
Centrifuge 5417 R	Eppendorf UK Limited, Stevenage, UK
Mini orbital shaker SO5	Bibby Scientific Limited, Stone, UK

Table 2-4 The equipment required for Magnetic Luminex® Assay

2.1.9 Immunofluorescence

Phosphate buffered saline tablets, polysine slides and cover glass and secondary antibody Alexa Fluor 594 dye (1:200) were obtained from Fisher Scientific, Loughborough, UK. Formaldehyde solution, bovine serum albumin, Triton x-100 were from Sigma-Aldrich Company Ltd, Dorset, UK. Vectashield antifade mounting medium with DAPI was from Vector Laboratories Ltd, Peterborough, UK. Nail hardener was obtained from Barry M Cosmetic, London, UK. Primary antibody anti-asc, pAb (AL177) (1:500) was purchased from Caltag Medysystems Ltd, Buckingham, UK and Kohler illumination from ZEISS, Cambridge, UK.

2.1.10 Molecular biology

2.1.10.1 Ribonucleic acid (RNA) extraction and quantification

RNase-free DNase I and RNeasy mini kits were both purchased from Qiagen, Manchester, UK.

Reagents	Unit
DNase I, RNase-Free (lyophilized)	1500Kunitz
Buffer RDD (DNase buffer)	2 x 2ml
RNase-Free Water	1.5ml

Table 2-5 Contents of RNase-free DNase 1 kit

Reagents	Unit
RNeasy Mini Spin Columns (pink)	250
Collection Tubes (1.5 ml)	250
Collection Tubes (2 ml)	250
Buffer RLT	220ml
Buffer RW1	220ml
Buffer RPE (concentrate)	65ml
RNase-Free Water	50ml

Table 2-6 Contents of RNeasy mini kit

Extra components used for the RNA extraction general protocol include Kimtech Science Kimwipes purchased from Kimberley Clark Professional, West Malling, Kent, England, UK and RNase Zap™ which was purchased from Sigma-Aldrich Ltd., Dorset, UK. Quantity and quality of RNA extraction was assessed by NanoDrop™ 8000 Spectrophotometer, ThermoFischer Scientific, Rugby, UK.

2.1.10.2 cDNA production

For the quantitative conversion of up to 2µg of total RNA to single-stranded cDNA, the high-capacity reverse transcriptase kit was used, Applied Biosystems Inc, California, USA. This product consists of 2 × 1mL of 10X RT Buffer, 2 × 1mL of 10X RT random primers, 1 × 1mL of 25X dNTP Mix (100mM) and 1 × 1mL of MultiScribe® Reverse Transcriptase (50 U/µL). Mineral oil was purchased from Sigma-Aldrich Ltd., Dorset, UK. The G-Storm, thermal cycler systems was from Agilegene Technologies Ltd., Somerset, UK.

2.1.10.3 Gel PCR

Agarose powder was purchased from Melford Biolaboratories Ltd., Ipswich, UK. TAE-diluted agarose gel was heated in the sensor microwave oven, (Panasonic, UK). GelRed™ nucleic acid gel stain, 10,000X was purchased from Biotium Inc., California, USA. Loading buffer blue (5X) was purchased from Bioline Reagents Ltd., London, UK. PCR sizer 100bp DNA ladder was purchased from NORGEN Biotek Corp., Ontario, Canada. All the gel trays, combs and tanks were purchased from Fischer Scientific, Loughborough, UK. The electrophoresis was run in TAE buffer (**Table 2-8**) using a power supply purchased from Bio-Rad Laboratories Ltd., Hertfordshire, UK. Gel was analysed using an imaged GeneGenius Gel Imaging System and printed out using a digital graphic printer, Syngene, Cambridge, UK.

Materials	Amount
Tris base	48.4g
Glacial acetic acid (17.4M)	11.4ml
EDTA, disodium salt	3.7g
Deionized water	Up to 1 L

Table 2-7 Recipe for 10X TAE electrophoresis buffer

2.1.10.4 Quantitative real-time PCR

Quantifast® SYBR® green PCR kit was purchased from Qiagen, Manchester, UK. Primers (RPL37A, ACTB and NLRP3) were obtained from Sigma-Aldrich Ltd., Dorset, UK. ViiA7 real-time PCR system was purchased by Applied Biosystems®, Life Technologies, California, USA.

2.2 Methods

2.2.1 Tissue culture

Prior to tissue culture, all the cells were kept frozen in ampoules in liquid nitrogen at early passage. The ampoules were thawed in a 37°C water bath for two minutes. Subsequently, the cells were transferred by 1ml Gilson pipette to a sterile tube, media added, then centrifuged, 125 rcf to give a cell pellet. The pellet was re-suspended and was transferred to the T75 flask and media added again to make up 10ml. Morphology of the cells was observed through the EVOS cell imaging system (Fisher Scientific, Leicestershire, UK).

THP-1 cells were derived from peripheral blood monocytes from a 1-year-old patient with acute monocytic leukemia as previously described (Tsuchiya et al., 1982). Cells were stored frozen in liquid nitrogen. Upon passaging, the cells were grown in ATCC-formulated RPMI-1640 medium containing 10% FBS and L-glutamine as amino acid supplement for the cells (Levintow et al., 1957). THP-1 cells are grown as suspension cultures. The cells were subcultured every 2 to 3 days.EA.hy926 cells (or somatic cell hybrid endothelial cells) were derived from human umbilical vein cell lines as previously described (Rieber et al., 1993). The base medium for these cells was ATCC-formulated Dulbecco's Modified Eagle's Medium containing 10% FBS for stimulating cell growth, proliferation and promoting differentiated functions (Brunner et al., 2010). Ea.hy926 cells were grown to approximate 80% confluency prior to passage. Cells were passaged by adding 2 ml trypsin/ EDTA (0.25%) for 1 to 2 minutes at 37ºC. Following neutralization with growth media, cells were centrifuged at 125g for 5 minutes. Cells were seeded at approximately $1X10^6$/ T25 flask for experimentation.

HUVEC cells were derived from multiple donors and each vial contains a minimum of 500,000 cells as previously described (Baudin et al., 2007). Upon passaging to the recommended T75 flask, the cells were grown in 15ml of human large vessel endothelial cell basal medium and 10ml of supplement. Subcultures of the cells were made during active division (that is 60-80% confluence). HUVEC were passaged as described above for EA.hy926 cells.

For cell counting, trypan blue and a haemocytometer were needed. Trypan blue or vital stain detects non-viable cells as blue, rounded and smaller in nature. Equal volume of cells and trypan blue were mixed (e.g. 10µl each) in a small Eppendorf tube and mixed for a few seconds. The mixed sample was drawn up into the space between the counting chamber and the coverslip. Viable cells were counted using the light microscope at low power.

Any unwanted cells were frozen again in liquid nitrogen after centrifugation to form the pellet. The cell pellet (between 1 to 4 X 10^6 cells/ml) was then mixed with the freezing medium, which consists of 50% serum (FBS), 40% medium and 10% dimethyl sulfoxide (DMSO) and 1ml aliquots made in freezing vials.

2.2.2 Cell treatment

THP-1, EA.hy926 and HUVEC cells were treated with PMA (5ng/ml) for 1,2 and 3 days (± bzATP) (Rajamaki et al., 2010), LPS (5µg/ml) for 1 day (± bzATP) (Sharma et al., 2010), bzATP (300µM) for 1 hour (Franceschini et al., 2015) and combined treatment of PMA followed by LPS (± bzATP) (Park et al., 2007).

For the detection of mitochondrial reactive oxygen species by intracellular superoxide measurement, THP-1 cells were diluted with 200nM MitoSox buffer solution at the end of the 30 minutes of experimentation. Subsequently, the MitoSox buffer solution was removed and the cells were washed with culture medium. Cells were observed with a fluorescence microscope using 569-nm excitation and 594-nm emission (Dikalov and Harrison, 2014).

HeLa cells were treated with etoposide (25µM) for 24 hours as a positive control/ for capsase-5 detection (Hitomi et al., 2004).

2.2.3 Protein extraction protocol

2.2.3.1 Radioimmunoprecipitation assay (RIPA) buffer

RIPA buffer is mandatory for tissue and cell lysis buffer for rapid, efficient and solubilization of proteins from both adherent and suspension cultured mammalian

cells while avoiding protein degradation and interference with the protein immunoreactivity and biological activity (Ji, 2010).

2.2.3.2 Protein extraction

Cells (confluency 80-99%) were detached from the T75 flask by trpysinization. Growth medium was added after the detachment of the cells for neutralizing the action of trypsin. The cells were then transferred to 15ml sterile tubes and centrifuged for 5 minutes, 20°C and 125 rcf for THP-1 cells, 125 rcf for EAhy926 cells and 136 rcf for HUVEC cells. After the centrifugation, the supernatant was removed to a waste bottle. The remaining cell pellet was washed for the second time with 1ml of PBS in 1.5ml Eppendorf tubes and centrifuged again. Subsequently, the supernatant was removed again and the pellet was mixed with phosphatase inhibitor and RIPA buffer with the ratio of 1:100 (e.g. 0.7µl of phosphatase inhibitor and 70µl of RIPA buffer) and kept on ice or at 4°C. The phosphatase inhibitor was needed for slowing down the process of dephosphorylation of the protein. The lysate was centrifuged at 4000 rcf, 4°C for 10 minutes. Finally, the supernatant was stored (typically 5µl to 10µl) in appropriately sized vials at -80°C.

2.2.4 Determining protein concentration with DC protein assay

DC protein assay is a colorimetric assay for protein concentration measurement following detergent solubilisation. The methods are previously well-described as ‘Lowry assay’ but have been modified to save time (Lowry et al., 1951). This method combines the reactions of copper ions with peptide bonds under alkaline conditions containing sodium potassium tartrate with the oxidation of aromatic protein residues.

For the preparation of a working standard curve, 5 dilutions of protein standard were used from 0 to 1.4µg/µl. Highest protein standard concentration (1.4µg/µl) was obtained from mixing 0.8ml bovine serum albumin concentrate with 0.34ml RIPA buffer.

Standard	[Protein] µg/µl	RIPA, µl	Pr Standard, µl
Standard 1	0	40	0
Standard 2	0.35	30	10
Standard 3	0.7	20	20
Standard 4	1.05	10	30
Standard 5	1.4	0	40

Table 2-8 Protein standard preparation

Protein standards and samples, 5µl each, were pipetted into a clean and dry 96 well plate in triplicate. The protein standard and samples were prepared in the same buffer (e.g. RIPA). Reagent A containing alkaline copper tartrate solution was added (25 µl) into each well of protein standard and sample. Subsequently, reagent B containing diluted Folin reagent was added (200µl) into each well. Any bubbles were popped out gently using a clean and dry pipet tip. The absorbance was read at 650nm wavelength after 15 minutes and was stable for one hour. The average absorbance value of the protein standards were plotted. The protein concentration of the samples was derived by interpolation.

2.2.5 Western Blot

Western blot is a well-known method for identifying proteins of interest by three steps: (1) molecular weight separation; (2) transferring or blotting to a membrane and (3) marking the target protein of interest by specific primary and secondary antibody (Mahmood and Yang, 2012). The complete process of Western blot can be further divided into 4 steps: gel preparation, electrophoresis, electro transfer and blocking and antibody incubation.

2.2.5.1 Gel preparation

Two types of gel were needed in the early part of Western blot. The first is a 12% resolving gel and overlaid with a 4% stacking gel. Resolving gel was basic (pH 8.8) as compared to stacking gel (pH 6.8), hence making the gel's pores narrower thus accurately separating the protein molecules. A stacking gel has lower acrylamide concentration hence making a more porous gel, separating proteins poorly but allowing them to form defined and sharp bands. A comb was inserted into the stacking gel without any trapped bubbles and left until the gel solidified (about 15 - 30 minutes later).

2.2.5.2 Electrophoresis.

When the gels were ready, the protein samples were loaded. Protein samples were mixed with loading buffer (equal volume), heated to 95°C for 5 minutes then straight away placed on ice. Glycerol is needed for easy sinking of the samples into the well. The bromophenol blue is also present to allow demarcating the progression of the separation. The sample was heated to allow the denaturing process of higher order structure while reducing disulphide bonds. Protein standard (5µl) was pipetted into the first well, followed by the samples in the remaining wells (maximum 50µl sample in each of 10 wells). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) 1X running buffer was poured into the gel tanks and electrophoresis performed at lower voltage (100 V) for 15 minutes and higher voltage (120 V) for 1 hour or until the dye ran out at the bottom of the gel.

2.2.5.3 Electrotransfer.

The process of electrotransfer is the mechanism for transferring the protein from the gel to the membrane using an electric field oriented perpendicular to the surface. There are two types of membranes which are commonly used; nitrocellulose and polyvinylidene fluoride (PVDF). However, PVDF membrane provides better mechanical support and allows reprobing with antibodies. A transfer sandwich was created as follows; sponge, 3 filter papers, gel, PVDF membrane, 3 filter papers and sponge. No air bubbles are ensured between membrane and gel, secured by roller. Transfer was for 90 minutes at 63V in cold transfer buffer (**Table 2-10**).

1 L of transfer buffer	Amount
Tris-base	3g
Glycine	13.3g
Methanol	100ml
dH_20 water	900ml

Table 2-9 Recipes for 1L of transfer buffer.

2.2.5.4 Blocking and antibody incubation

After all the proteins were transferred to the membrane, the membranes were blocked to prevent any non-specific antibody binding. Blocking was done with 5% bovine serum albumin (BSA) or 5% skimmed milk in Tris-buffered saline tween-20 (TBST) for one hour. Skimmed milk was the preferred blocking agent because it is inexpensive and widely available. Primary antibody in 5% skimmed milk was added and incubated overnight at 4°C on the rocker. Subsequently, secondary antibody in 5% skimmed milk was added and incubated for one hour at room temperature, on the rocker. Vigorous washing with TBST was needed before and

after the secondary antibody (3X 15 minutes). The washing was very important to remove unwanted antibodies but it should not left too long, as it may reduce the signals. The membrane was then incubated in ECL mixed reagent; equal volume of Reagent A (luminol solution) and Reagent B (peroxide solution) for 5 minutes and can be visualized with the ImageQuant analyser.

2.2.6 ELISA

Magnetic Luminex assay was performed to analyse active IL-1β and active lL-18 in the cell culture supernatant. These specific antibodies are pre-coated onto color-coded magnetic microparticles. Microparticles, standards and samples were pipetted into wells and the immobilized antibodies allowed to bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest was added to each well.

Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, was added to each well. A final wash removes unbound Streptavidin-PE and the microparticles were resuspended in buffer and read using the Luminex MAGPIX analyzer.

The magnet in the analyzer captures and holds the super paramagnetic microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and the second LED determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound. Each well was imaged with a CCD camera.

2.2.6.1 Sample preparation

Cell culture supernatant samples required at least a 2-fold dilution; 75µL of sample + 75µL of calibrator.

2.2.6.2 Reagent preparation

Wash buffer concentrate was added to distilled water to prepare 500ml of wash buffer. Each standard cocktail was reconstituted with calibrator diluent to make up a 10X concentration. The standards were provided lyophilised and were reconstituted with pre-specified volume of calibrator diluent RD6-52, as described in the 'Certificate of Analysis'. Standards' concentrations were variable between kit batches. For this analysis, six serial dilutions (1:2 in calibrator diluent RD6-52) were prepared in 300µl, each.

2.2.7 Immunofluorescence of ASC

All the treated and untreated THP-1 cells were incubated in 6 well plates. One cover glass was lay over on the bottom of the flask for each well plate. After all the treatment's duration were completed, the medium was removed and all the cover glasses inside the 6 well plates were washed with PBS adequately. Subsequently, 2ml of 4% formaldehyde/ PBS were added at room temperature, as a fixing solution. After 30 minutes, all the cover glasses in the well were washed three times with 2ml of PBS/ 0.5% BSA at room temperature. Following this, permeabilization was performed by adding 2ml of PBS/ 0.5% BSA/ 0.1% Triton X-100 at room temperature for 5 minutes. After washing, all the cover glasses were incubated with primary anti-ASC antibody solution (in BSA) for 1 hour at room temperature. Later, secondary antibody was introduced for 30 minutes at room temperature after adequate washing. Following washing, all the slides were taken out upside down and attached together with polysine slides with mounting medium. Nail vanish was applied at the edge of the cover glasses to secure the seal. The slides were ready to read through Kohler illumination after 1 hour (Compan et al., 2015).

Three repeats of each experiment were performed. Fifteen fields with more than 15 cells each were visualized. ASC-speck like pattern was counted and ratio of stained cells to total cells was calculated.

2.2.8 Molecular biology

2.2.8.1 RNA extraction

After medium was removed, all the adherent cells on the T-25 flask were washed thoroughly with PBS to remove unwanted or dead cells. For the homogenization step, RNA lysis buffer, 600µl (Buffer RLT, Qiagen) and β-mercaptoethanol were added to lyse the adhered cells. 10µl β-mercaptoethanol per 1 ml Buffer RLT was used. Finally, the lysate was kept either on ice or in a -80°C freezer to continue the extraction.

Prior to phase separation step, the working station was sprayed with RNAaseZAP to destroy any contamination with RNAases. Ethanol (70%) (600µl) was added and mixed thoroughly into the cell lysate at room temperature. Mixed ethanol-lysates were added into each column and centrifuged at 8000g for 30 seconds at room temperature. The flow-through was discarded.

Subsequently, in a DNase digestion step, RW-1 buffer was added to remove the protein from the cells in the column by protease degradation. The column was centrifuged at 8000 rpm for 30 seconds. DNase 1 incubation mix was added subsequently and centrifuged at 10000 rpm for 30 seconds. The remaining flow-through was discarded also.

Afterwards, in a desalting step, 500µl of buffer RPE were added to each column and centrifuged at 10000 rpm for 15 seconds followed by 2 minutes and finally centrifuged for 13000 rpm for 1 minute to further eliminate the waste. Finally, 35µl RNase-free water was added to reduce the risk of RNA being degraded by RNases. The column was centrifuged at 13000 rpm for 1 minute and left for 5 minutes on ice.

Each sample was read by NanoDrop 8000 spectrophotometer for assessment of the purity and concentration of the nucleic acids in samples. The software calculated the concentration as a 10mm absorbance path for convenience, displaying the concentration in ng/µl. The purity ratios indicated the quality of the samples. The purity of the RNA was determined by calculating the ratios for

OD260/280 (260nm- peak nucleic acid absorbance, 280nm- peak protein absorbance) and OD260/230 (260nm- peak nucleic acid absorbance, 230nm- peak carbohydrates, peptides, phenols, aromatic compounds absorbance).

A ratio of between 1.6 and 2.2 was classified as the acceptable range for 260/280, indicating RNA relatively free from protein contamination. For 260/230, a ratio of between 1.8 and 2.2 was the acceptable range and demonstrated a low level of contamination from molecules that absorb in the 230nm range.

2.2.8.2 cDNA synthesis

For 2µg RNA for each sample (8µl), 2µl of 50ng/µl random hexamer was added to each sample. Subsequently, 1µl of 100mM dNTP was added. After incubate in room temperature for 10 minutes, reverse transcription master mix was assembled (**Table 2-10**) and was added in each sample.

All the samples in the strip tubes were processed by G-Storm, thermal cycler system. Three steps were required for reverse transcription (**Table 2-11**). These three steps were repetitively continued for 30 cycles.

Reagent	µl
10X reaction buffer	2
RNase in or RNase out	1
Multiscripts	1
MilliQ water	6

Table 2-10 Reverse transcriptase master mix.

Step	Temperature, °C	Duration, min
Large cDNA synthesis	37	120
Heat inactivation	70	15
Dissociation of RNA	85	5

Table 2-11 Temperature setting for cDNA synthesis

2.2.8.3 Analysis of PCR products

An agarose gel was made by mixing 2-3g agarose powder with 100ml of TAE (mixture of Tris base, acetic acid and EDTA) water in a 500ml Duran bottle. Gel red (10µl) was added into the solution as an intercalating nucleic acid stain. After boiling for 4 minutes in a 900W microwave, the mixture was poured on a 16-tooth gel tray and left for 30 minutes for the gel to set. After the gel set, the combs were removed slowly and the gel immersed in a 1X TAE buffer in the tanks until well-filled and submerged. To each strip tube was added 4µl of 5X loading buffer and all of the sample was gently loaded into the well. Electrophoresis was performed at 120V for 45 minutes. The bands on the gel were visualized on the GeneGenius Gel Imaging System and printed out on a digital graphic printer.

2.2.8.4 Quantitative real-time PCR

Generally, quantification of DNA is performed by targeting the progressing amplification of the target sequence by linking to fluorescence intensity via fluorescent reporter molecule (Fraga et al., 2014). In real time qPCR, the fluorescence is measured at each cycle as the amplification progresses, by DNA binding dyes such as SYBR Green I which is highly cost effective and easy to use (Ponchel et al., 2003). Principally, threshold cycle or (C_T) value is analysed by plotting the fluorescence versus cycle number for all the samples. C_T value is calculated from an amplification plot as the point where the plot crosses the threshold fluorescence. The greater amount of initial DNA template, the earlier the C_T value (Schmittgen and Livak, 2008) (**Figure 2-1**).

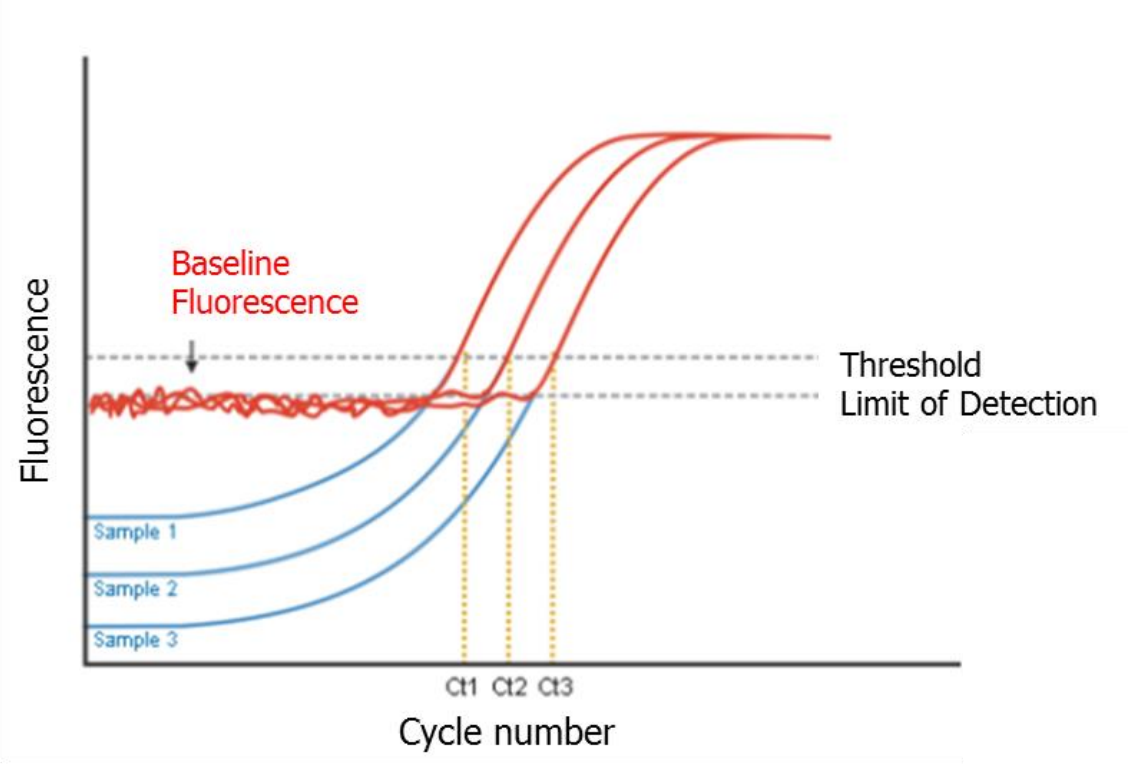

Figure 2-1 Principles of real-time qPCR

Schematic diagram demonstrates the measurement of C_T. Threshold level of fluorescence is set above the baseline fluorescence. C_T value is correlated to the starting target concentration of sample. In this example, Sample 1 has the highest DNA concentration and Sample 3 the lowest.

SYBR Green I is a non-specific DNA binding dye that fluoresces when bound to double-stranded DNA (Dragan et al., 2012). **Table 2-12** details the SYBR Green I qPCR reaction mixture for each 20µl sample.

Reagent	X 1 reaction
2 X SYBR mix	10µl
DMSO	1µl
Forward primer (10µM)	0.5µl
Reverse primer (10µM)	0.5µl
H_2O	7µl
cDNA	1µl

Table 2-12 qPCR reaction mixes.

Table 2-13 gives a detailed list of primers used for each gene of interest and endogenous housekeeper genes:

No	Primer	Sequence (5' to 3')	Annealing Position	Amplicon length
1	***NLRP3***			
	Forward	CATCGGGTGGAGTCACTGTC	2766-2785	90 bp
	Reverse	AAGGTGTCGGCCTTCCTTTT	2830-2849	
2	***NLRP3***			
	Forward	CAAACGCTACACACGACTGC	1241-1260	175 bp
	Reverse	CATCGGGGTCAAACAGCAAC	1364-1383	
3	***RPL37A***			
	Forward	GGCGACATGGCCAAACGTACC	73-93	120 bp
	Reverse	AGTGTACTTGGCGTGCTGGCT	110-130	
4	***RPL37A***			
	Forward	ACAATACCACTTCCGCTGTC	290-310	100 bp
	Reverse	CCAGTGATGTCTCAAAGAGTAGAG	364-388	
5	***ACTB***			
	Forward	GGATCAGCAAGCAGGAGTATG	1259-1280	100 bp
	Reverse	AGAAAGGGTGTAACGCAACTAA	1333-1355	
6	***ACTB***			
	Forward	TCCACCGCAAATGCTTCT	1301-1319	100 bp
	Reverse	AGCCATGCCAATCTCATCTT	1382-1402	

Table 2-13 List of primers of gene of interest and housekeeping genes.

The comparative C_t method was used to quantify or analyse the amount of PCR product in each sample using a previously reported method of analysis (Livak and Schmittgen, 2001).

2.2.9 Data analysis and statistical testing

All data are presented as mean and standard error of mean (SEM). Statistical analyses were performed using GraphPad® Prism 7 Software (GraphPad Software, Inc., CA, USA). Statistical analysis was performed to determine distribution of data using normality test and it was found to be normally distributed. The cases were independent of each other and homogenous in nature.Statistical significance was determined by applying one-way ANOVA with an appropriate post-hoc test for comparison of three or more groups or Student's *t*-test for the comparison of 2 groups. A p-value of <0.05 was considered evidence of statistical significance.

Chapter 3 NLRP3 inflammasome Signal 1 and Signal 2 in THP-1 cells and endothelial cells

3.1 Background

3.1.1 Signal 1 and Signal 2 of the NLRP3 inflammasome

The NLRP3 inflammasome is one of the innate host defence mechanisms in response to PAMPS or DAMPS (Iwasaki and Medzhitov, 2010). Infection, metabolic disorders and tissue damage trigger NLRP3 inflammasome formation (Schroder and Tschopp, 2010). This active multiprotein complex prevents further tissue damage by inducing programmed cell death; apoptosis or pyroptosis (Sagulenko et al., 2013). However, over stimulation of the inflammasome may lead to auto inflammatory disease and host tissue damage (Davis et al., 2011). Thus, the balance between activation and inhibition of NLRP3 inflammasome production must be preserved to remove unwanted pathogens whilst being harmless to the host (Latz et al., 2013). Two steps of activation are needed to form the active inflammasome complex; Signal 1 or priming, followed by Signal 2 or activation.

Priming agents such as LPS are recognised by TLRs, located on the cell membrane. LPS is one of the bacterial cell wall components. TLR recognize PAMPs or microbes by the presence of germ-line encoded PRR (pattern recognition receptors) (Mariathasan and Monack, 2007). Subsequently, the TLR activates transcription factor NF-κB, which is located in the cytoplasm. Furthermore, signalling via NF-κB pathways promotes transcription and translation of many inflammasome components including NLRP3, pro-IL-1β and pro-IL-18 which accumulate inside the cytosol (Strowig et al., 2012).

Signal 1 itself is insufficient to produce the active inflammasome. Hence the inflammasome components need to undergo oligomerization which is activated in response to potassium efflux, calcium influx, lysosomal damage and mitochondrial dysfunction (Sutterwala et al., 2014). Extracellular ATP triggers the Signal 2 cascade by activating the $P2X_7$ receptors of the cell membrane hence promoting potassium efflux (Di Virgilio, 2007).

The oligomerization of inflammasome components leads to formation of active caspase-1 by proteolytic activation of pro-caspase-1 (Mariathasan and Monack, 2007). Caspase 1 acts as an effector component of this multiprotein complex and is also known as IL-1β converting enzyme which is necessary for the production of mature IL-1β as well as mature IL-18 in the cytoplasm. These end products of the active NLRP3 inflammasome (Signal 2) cause the inflammatory response of cells following secretion from the cell of origin (Yu and Finlay, 2008). Furthermore, caspase-1 activation leads to HMGB1 (high mobility group box 1) release by an IL-1β-independent process either directly or indirectly by HMGB1 translocation from the nucleus to the cytoplasm (Lu et al., 2012). HMGB1 activates RAGE (receptor for advanced glycation end products) for further IL-1β and TNF-α release (Willingham et al., 2009).

3.1.2 NLRP3 inflammasome in THP-1 cells

THP-1 cells are human cancer cells derived from a patient with acute monocytic leukemia. The monocytic features of this cell include asymmetrical morphology, bean-shaped nucleus and granulations within the cytoplasm (Chanput et al., 2014). THP-1 is considered as an early stage of the monocyte lineage that are involved in innate immune response. Differentiation of monocytes into macrophages is considered one of the crucial steps *in vivo* in the defence against pathogens. Phorbol-12-myristate-13-acetate (PMA) is a common *in vitro* stimulus for differentiation to macrophages (Daigneault et al., 2010). The differentiated THP-1 macrophage-like cells are adherent with larger bean-shaped nuclei and a thinner peripheral rim of heterochromatin (Van der Rhee et al., 1979).

Even though both monocytes and macrophages have a common specific cell surface marker which is CD14, macrophages show a higher affinity CD14 which is a receptor of complexes of LPS and LPS binding protein (Aldo et al., 2013). Studies showed that in differentiated THP-1 cells LPS promotes the activation of Signal 1, evidenced by the expression of NLRP3 protein and pro-inflammatory markers such as pro-IL-1β and pro-IL-18 (He et al., 2012).

There are some advantages of investigating THP-1 cells as compared to human monocytic cells, including generally less phenotypic variations due to the

background homogeneity in THP-1 cells, which is important for studying basic biological functions (Qin, 2012). However, specific treatments are required to promote the formation of active NLRP3 inflammasome. Upon Signal 2 activation by extracellular ATP, it is believed all eukaryotic cells, such as differentiated THP-1 cells,show potassium efflux extracellularly, mitochondrial perturbation and lysosomal damage . Subsequent production of mitochondrial ROS activates the de-ubiquitination of NLRP3 protein and further formation of the multiprotein complex of the active inflammasome which results in production of activated caspase-1 (Haneklaus et al., 2013).

3.1.3 Endothelial cells and inflammasome

Endothelial cells line the innermost surface of blood vessels and have multiple functions (Wu and Ringeisen, 2010). Studies suggest that the most important function is as a physical barrier towards pathogens. Nevertheless, there are studies demonstrating the relevance of inflammation in endothelial cells (Lin et al., 2014, Song et al., 2015, Wang et al., 2011, Wu et al., 2014). Endothelial cells contain IL-1 and components of the inflammasome, including apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1, and NLRP3. Somehow, during acute inflammation, endothelial cells release important inflammatory mediators such as leukotrienes, major histocompatibility complex (MHC) and interleukins (Muczynski et al., 2003).

As the name implies, human umbilical vein endothelial cells (HUVEC) are derived from the vein of umbilical cord. HUVEC are commonly studied due to their availability, hence have been used to demonstrate the major role of endothelial cells in regulation of responses towards stress (Park et al., 2006). Moreover, HUVEC have been shown to be responsive after treatment with LPS, high glucose and shear stress (Cao et al., 2017). Furthermore, HUVEC express crucial endothelial markers such as ICAM-1, VCAM-1, selectins and nitric oxide (Kim et al., 2001).

However, there are some limitations upon handling HUVEC such as the presence of contaminating cells, slow growth and progressive loss of cell markers and viability.

Besides studying primary endothelial cells such as HUVEC, one of the other common cells utilised is the EA.hy926 cell line (Edgell et al., 1983). It is derived from a fusion between HUVEC and A549 lung carcinoma cells and displays endothelial markers such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Baranska et al., 2005).

As mentioned above, many eukaryotic cells manifest some inflammatory responses, hence, endothelial cells may also express NLRP3 inflammasome activation during inflammation. A previous *in vitro* study also demonstrated the presence of NLRP3 inflammasome components in cultured mouse microvascular endothelial cells, including NLRP3, ASC and caspase-1 (Xia et al., 2014). In addition, a study in human retinal endothelial cells also showed Signal 2 inflammasome end products, IL-1β via TXNIP-NLRP3 interaction (Mohamed et al., 2014).

3.2 Aims

Previous unpublished data from this group showed that *in vitro,* mitochondrial dysfunction induced a pro-inflammatory endothelial phenotype. It was hypothesised that the mechanism might involve changes to inflammasome activation involving mitochondrial redox biology or release of mitochondrial DAMPs. However, before proceeding with any treatments that disrupt endothelial cell mitochondrial function, the basic inflammatory NLRP3 inflammasome expression, priming and activation in human endothelial cells was investigated and contrasted with NLRP3 inflammasome activation in THP-1 cells.

The aims of this chapter were:

- to demonstrate NLRP3 inflammasome Signal 1 in THP-1 cells, as a positive control cell type
- to show Signal 2 activation of NLRP3 inflammasome in THP-1 cells as a positive control
- to investigate NLRP3 inflammasome Signal 1 in human endothelial cells
- to investigate Signal 2 pathways in human endothelial cells

3.3 Experimental design

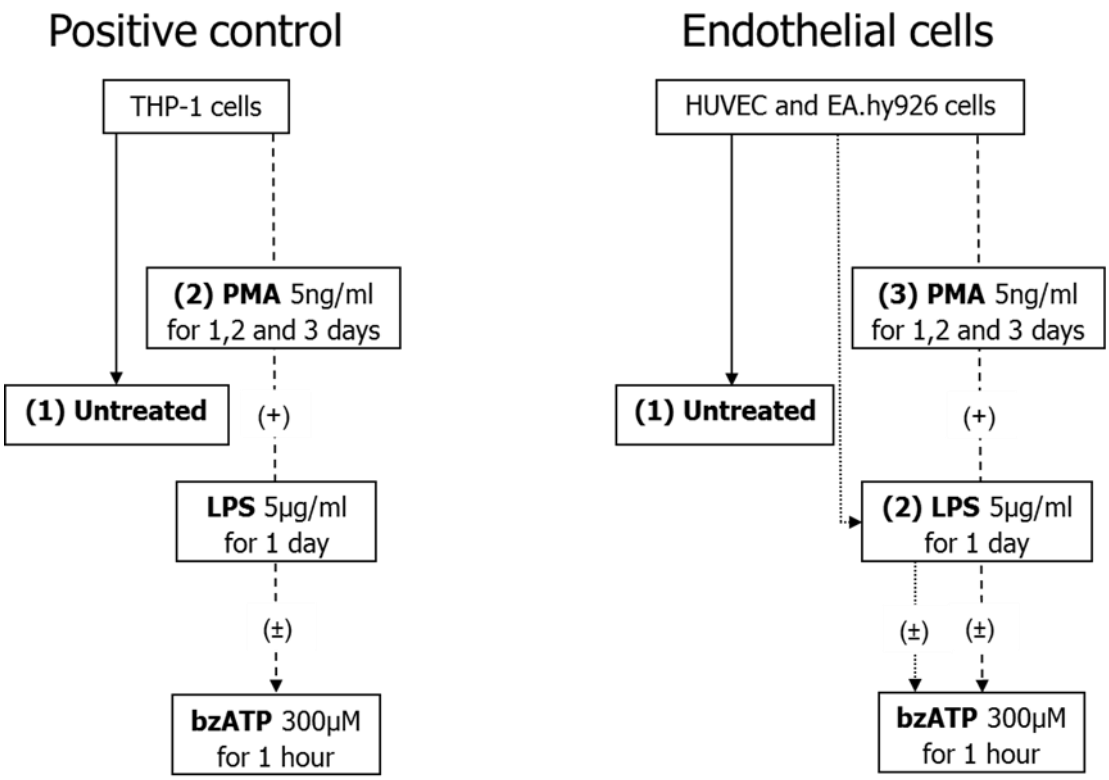

Figure 3-1 Flow chart showing research outline for the study.

Flow diagram shows step-by-step treatments of THP-1 cells and endothelial cells.

3.4 Results

3.4.1 Effect of PMA on THP-1 cells morphology

Untreated and treated THP-1 cells were observed via light microscopy (LM). Untreated THP-1 cells showed single, spherical and floating cells- typical of monocytic cell types (**Figure 3-2**). Subsequent differentiation by PMA showed macrophage-like features such as adherence to the plastic surface, spreading and amoeboid shape (Chanput et al., 2014). Less cells were observed after LPS+bzATP administration suggesting dying cells due to apoptosis but the remaining cells showed no further morphological changes.

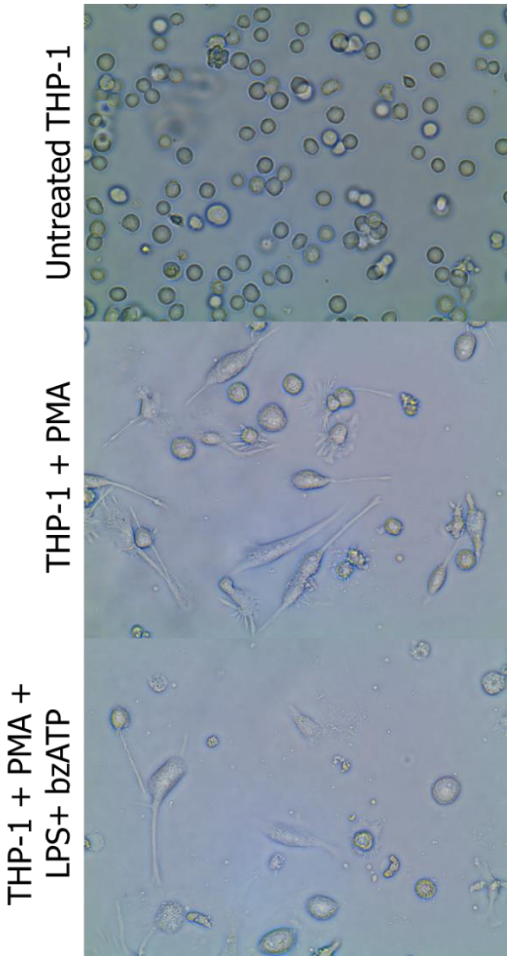

Figure 3-2 Photomicrograph showing the morphology of THP-1 cell.

Differentiated THP-1 cells with PMA exhibit attached cells to the flask, flattened, amoeboid shape and increase cytoplasmic: nucleus ratio. However, there were no obvious further morphology changes upon LPS+bzATP administration. LM X 40.

3.4.2 NLRP3 inflammasome Signal 1 and Signal 2 pathways in THP-1 cells

As illustrated in **Figure 3-2**, PMA (5ng/ml) was used to differentiate the THP-1 cells to macrophages. Subsequently, LPS (5µg/ml) was added for 24 hours and cells were harvested and lysed for Western blot analysis. Cell lysate analysis by Western blot revealed Signal 1 activation in all samples of PMA and LPS treated THP-1 cells (**Figure 3-3 and Figure 3-4**). There was no obvious difference in expression of NLRP3, pro-IL-1β and pro-IL-18 with extended exposure to PMA for 2 or 3 days duration compared with a single day. Hence, for subsequent investigations, THP-1 cells were treated with PMA for 24 hours to allow differentiation into macrophages and expression of inflammasome components.

Cell culture media were used to assess IL-1β and IL-18 synthesis and release from THP-1 cells and these were measured using Magnetic Luminex Assay, ELISA (**Figure 3-5**). Untreated and treated THP-1 cells were analysed in 3 repeats. Results showed increased IL-1β and IL-18 release upon treatment with bzATP as compared to without bzATP. This indicated that bzATP is a Signal 2 stimulator for the NLRP3 inflammasome. However, there were no significant differences between cells differentiated with PMA for 1, 2 or 3 days respectively. As expected, no IL-1β and IL-18 were released in untreated THP-1 cells suggesting quiescent THP-1 cells did not possess features of professional macrophage cells.

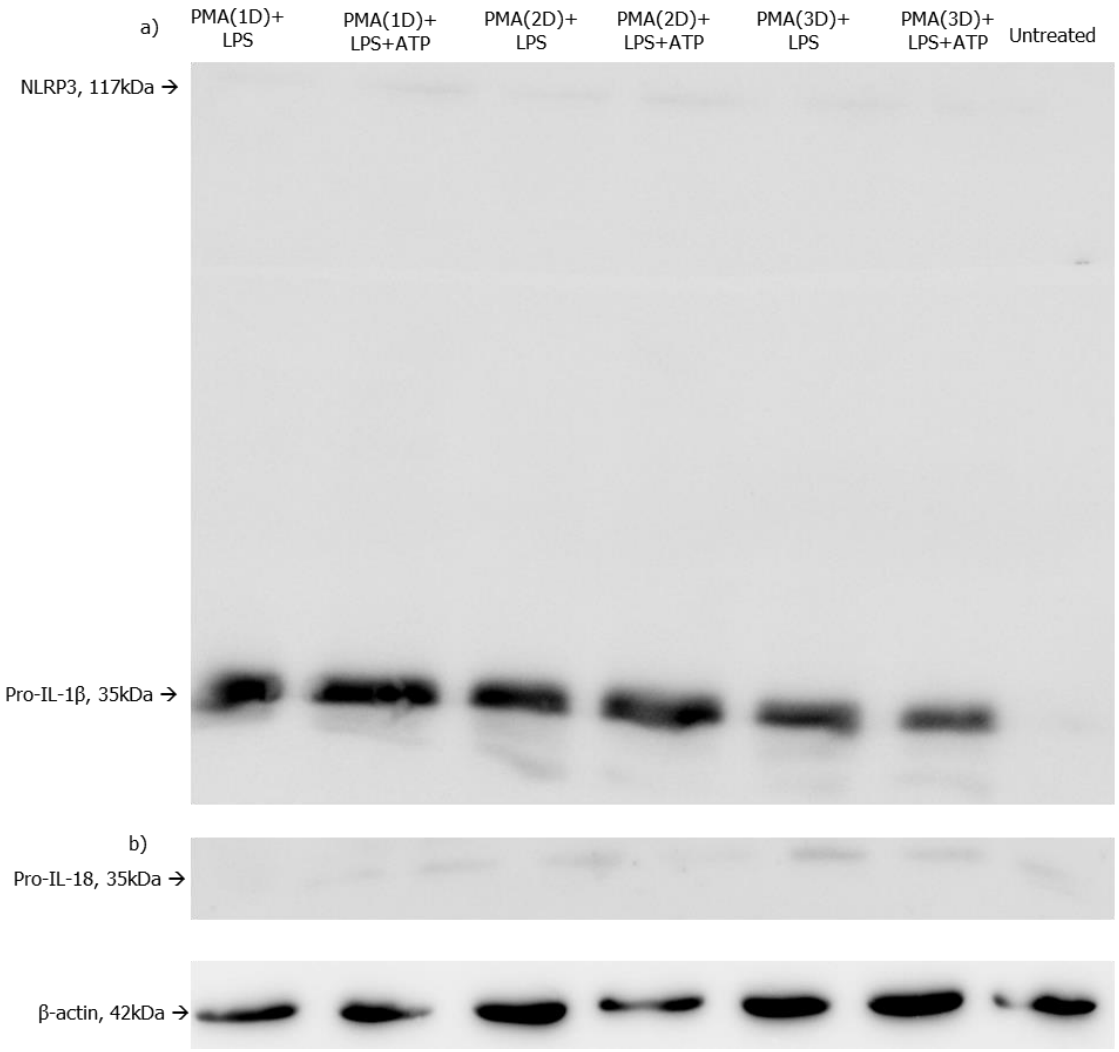

Figure 3-3 Signal 1 and Signal 2 expression in THP-1 cells.

Western blot showing the expression of NLRP3, pro-IL-1β and pro-IL-18 (Signal 1) in differentiated treated THP-1 cells. PMA was used for 1, 2 and 3 days to differentiate monocytes to macrophages followed by LPS 5µg/ml (for Signal 1 activation) ± bzATP 300µM for Signal 2 activation. Pro-IL-1β (**a**) and pro-IL-18 (**b**) bands were detected with specific antibodies. No bands were visualized in untreated THP-1 cells.

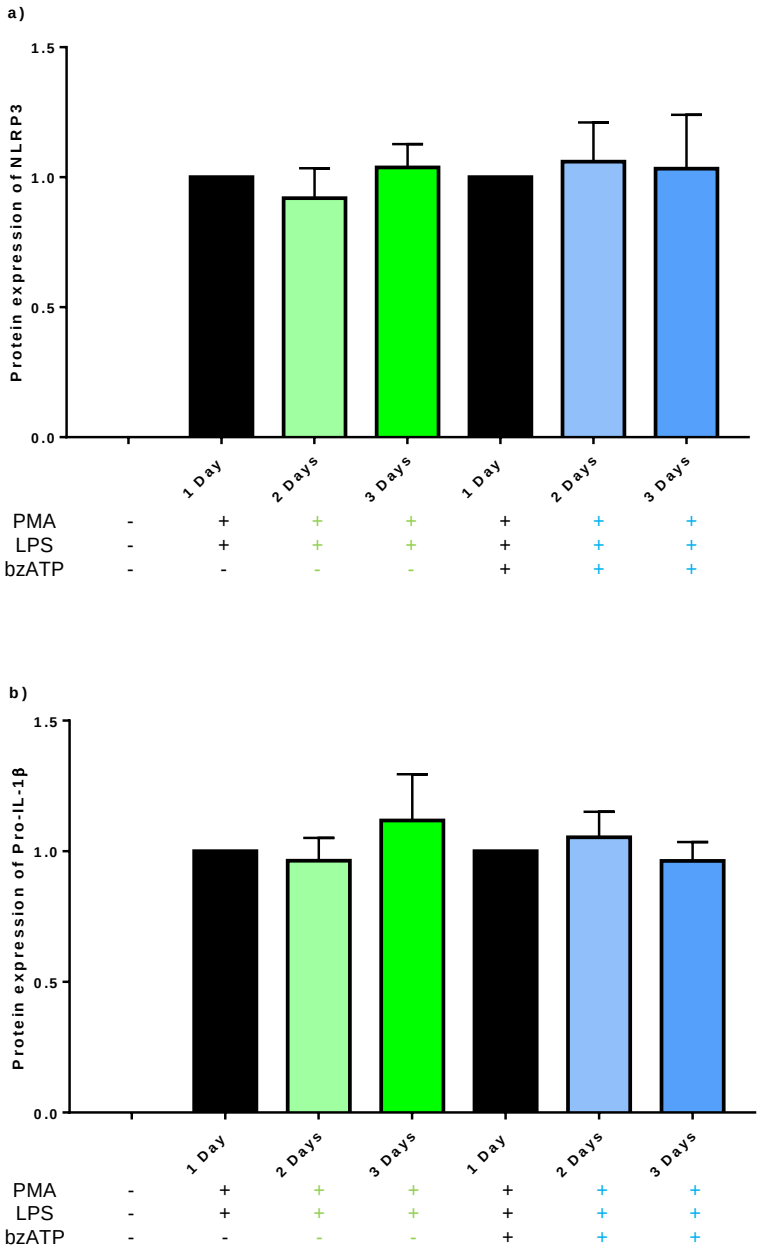

Figure 3-4 Quantitation of Signal 1 expression in THP-1 cells.

Quantitative analysis from Western blot (Figure 3-1) showing the expression of Signal 1; NLRP3 and pro-IL-1β, relative to β-actin PMA was used for 1, 2 and 3 days to differentiate monocytes to macrophages followed by LPS 5µg/ml (for Signal 1 activation), with or without bzATP. No differences in NLRP3 (a) and pro-IL-1β (b) expression either 1, 2 or 3 days of PMA, with or without bzATP were observed. Data presented as mean ± SEM, n=3, relative to β-actin, as compared to 1 day treatment respectively. Statistical analysis (ANOVA) was used to compare cells treated for 2 and 3 days versus 1 day treatment.

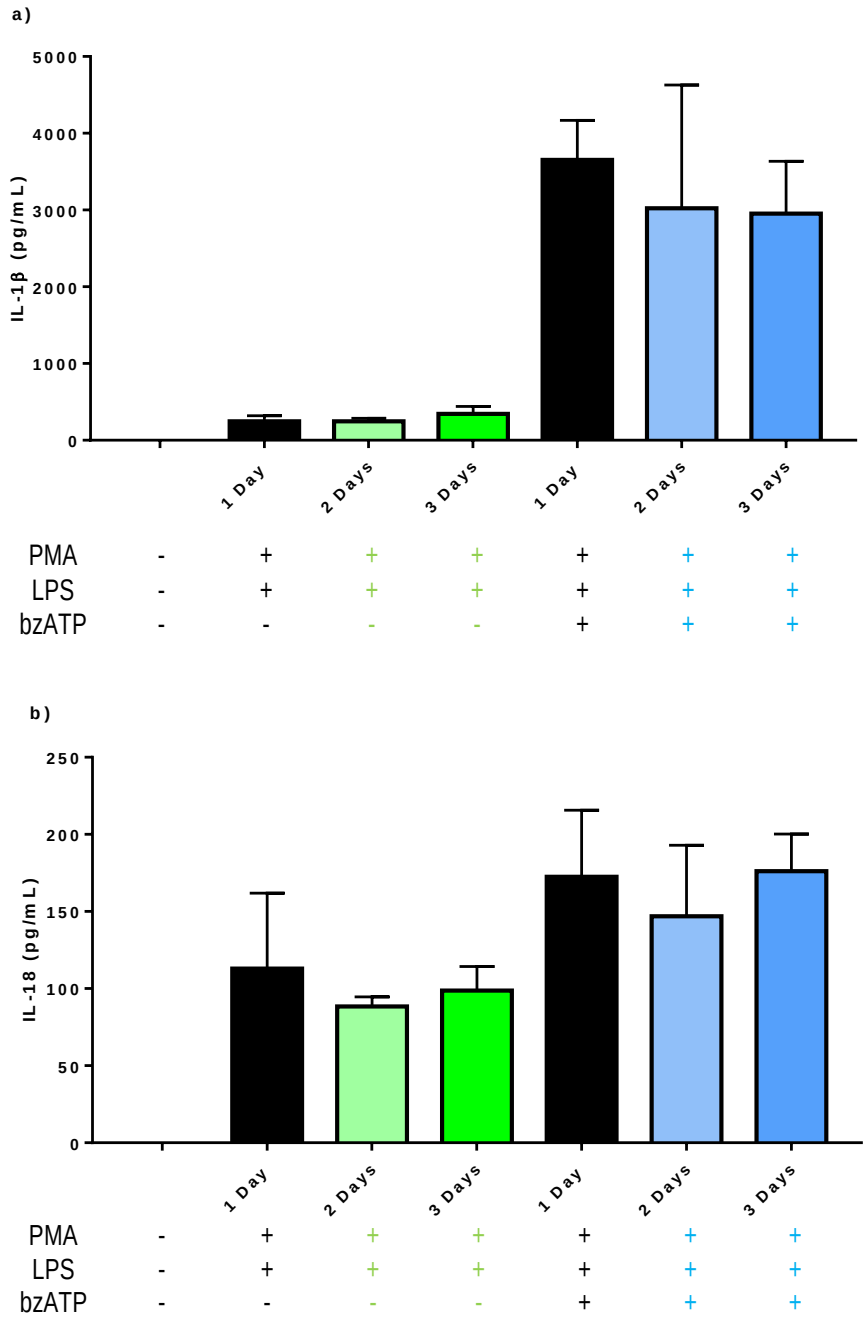

Figure 3-5 Signal 2 expression in cell culture supernatant of THP-1 cells.

Media from experiments shown in **Figure 3-1** were analysed for active IL-1β (**a**) and IL-18 (**b**) release by ELISA. As expected, bzATP acted as a potent Signal 2 activator evidenced by higher IL-1β and IL-18 released as compared to cultures without bzATP administration. However, no significant differences were observed when comparing cells treated with PMA for 1, 2 or 3 days. Data are presented as mean ± SEM, n=3. Statistical analysis (ANOVA) was used to compare cells treated for 2 and 3 days versus 1 day treatment.

3.4.1.1 Effect of LPS concentration on priming of the THP-1 NLRP3 inflammasome

A wide range of concentrations of LPS has been used in the literature to activate the NLRP3 inflammasome in differentiated THP-1 cells. Therefore, a range of concentrations was investigated with respect to Signal 1 (pro-IL-1β) expression following PMA differentiation for one day (**Figure 3-6**). There was no clear concentration-dependent effect of LPS when comparing pro-IL-1β expression for 0.1, 1 and 5µg/ml LPS for 1 day. Hence, it is concluded that higher concentrations of LPS might not increase Signal 1 in THP-1 cells and therefore that the lowest concentration could be used in subsequent experiments. Additionally, a previous study demonstrated that excessive secretion of TNF-α by LPS causes endotoxic shock hence masking the inflammasome effect (Wright et al., 1990).

Furthermore, inflammasome priming was assessed by the presence of the ASC component of the inflammasome. The release of active IL-1β is dependent on this adaptor component of the inflammasome complex (Hara et al., 2013). Cytosolic macromolecular aggregation of ASC dimer (ASC specks) occurs during inflammation hence recruiting pro-caspase-1. Subsequently, the induction of pyroptosis by mature caspase-1 releases IL-1β and IL-18 (Bryan et al., 2009). As expected, LPS triggered ASC-speck like formation (**Figure 3-7**). However, less ASC-speck like formation was observed when bzATP was added. It is presumed that the end products (IL-1β and IL-18) are secreted into the cell media and the inflammasome is de-assembled (see Discussion).

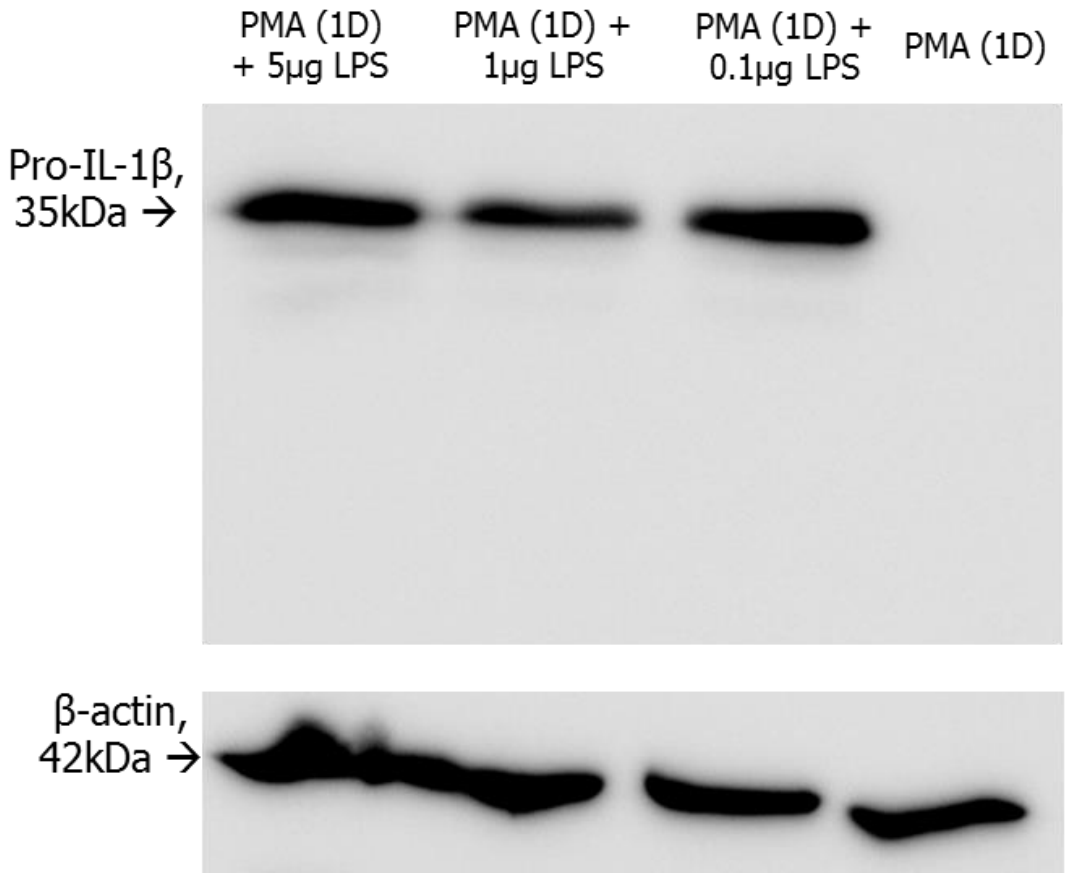

Figure 3-6 Signal 1 expression in difference concentrations of LPS in THP-1 cells.

Western blot showing the expression of pro-IL-1β (Signal 1). PMA was used to differentiate monocytes to macrophages followed by administration of different concentrations of LPS (0, 0.1, 1 and 5µg/ml). However, no obvious difference in expression of pro-IL-1β was observed when comparing each concentration of LPS, n=1.

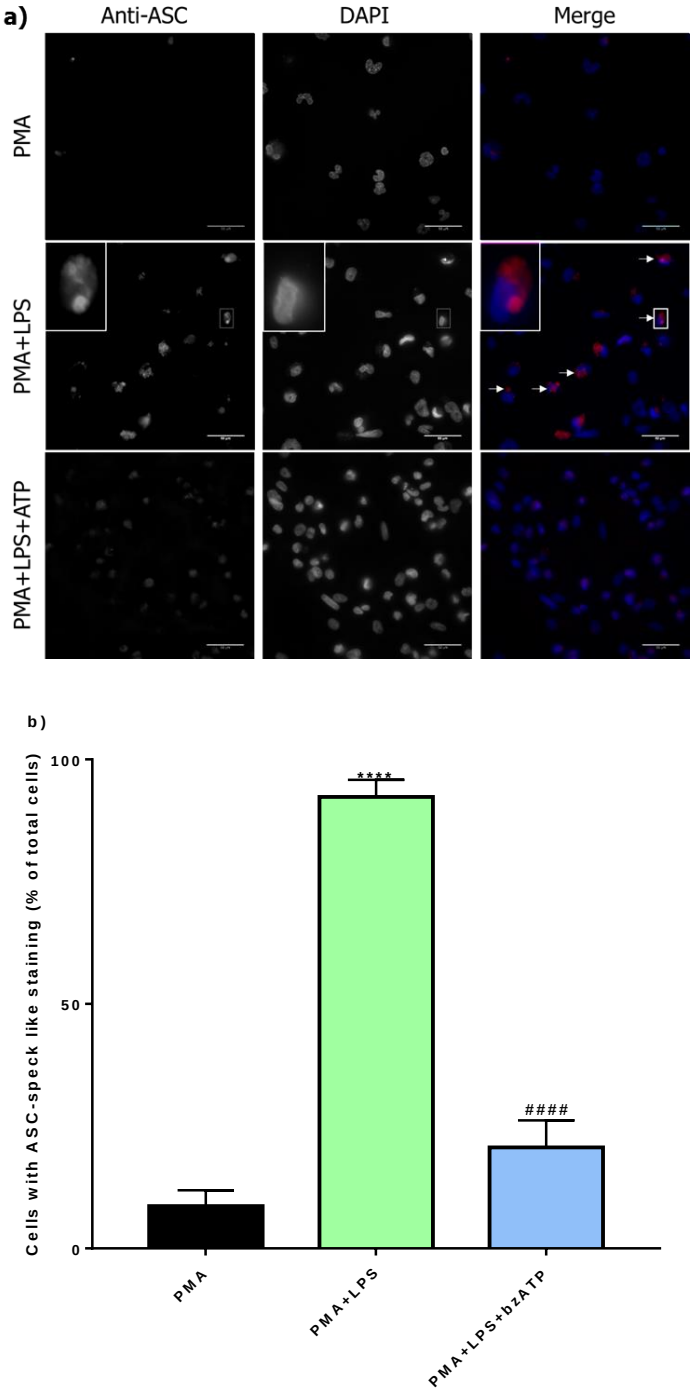

Figure 3-7 Immunofluorescence of ASC-specks

THP-1 cells were treated with 5ng/ml PMA for 24 hours, followed by 0.1µg/ml LPS (with or without bzATP). Speck like aggregates of ASC were observed by immunofluorescence microscope (white arrows, red fluorescence) (**a**). PMA+LPS treatment show an increase of ASC-speck staining indicating a robust inflammasome priming process. Scale bars, 50 µm. Image shown at 40X magnification. Inset shown at 8X magnification. Data presented as mean + SEM, n=3 (****p<0.0001 as compared to PMA alone and ####p<0.0001 as compared to PMA+LPS) by ANOVA (**b**).

3.4.3 NLRP3 inflammasome priming and activation pathways in HUVEC

PMA 5ng/ml was used for 1, 2 or 3 days for comparison with THP-1 cells. This was followed by LPS (5µg/ml) for Signal 1 and then bzATP (300µM) for Signal 2 activation. In addition, HUVEC were also treated without PMA to further investigate these effects. Western blot for NLRP3 and its quantitation in untreated and treated HUVEC was performed (**Figure 3-8** and **Figure 3-9)**. Three antibodies were used on these two blots; anti-NLRP3, anti-IL-1β and anti-IL-18. Results showed the presence of NLRP3 inflammasome components (NLRP3 and pro-IL-18) following PMA and LPS stimulation of HUVEC, regardless of the presence of bzATP. However, no pro-IL-1β expression was visualized in any of the samples. Furthermore, much lower expression of pro-IL-18 and NLRP3 was noted in any of the HUVEC treatments without PMA suggesting PKC activation by PMA could be involved. In addition, there was no obvious difference in expression of NLRP3 protein over the PMA treatment time course.

Release of IL-1β and IL-18 by HUVEC into cell culture media was measured by Magnetic Luminex Assay ELISA. Untreated and treated HUVEC were analysed in 3 repeats. Additionally, HUVEC also were treated with LPS without PMA. However, both IL-1β and IL-18 release was similar to the untreated cells in all treatment types indicating background levels and therefore no inflammasome activation in these cells (**Figure 3-10**).

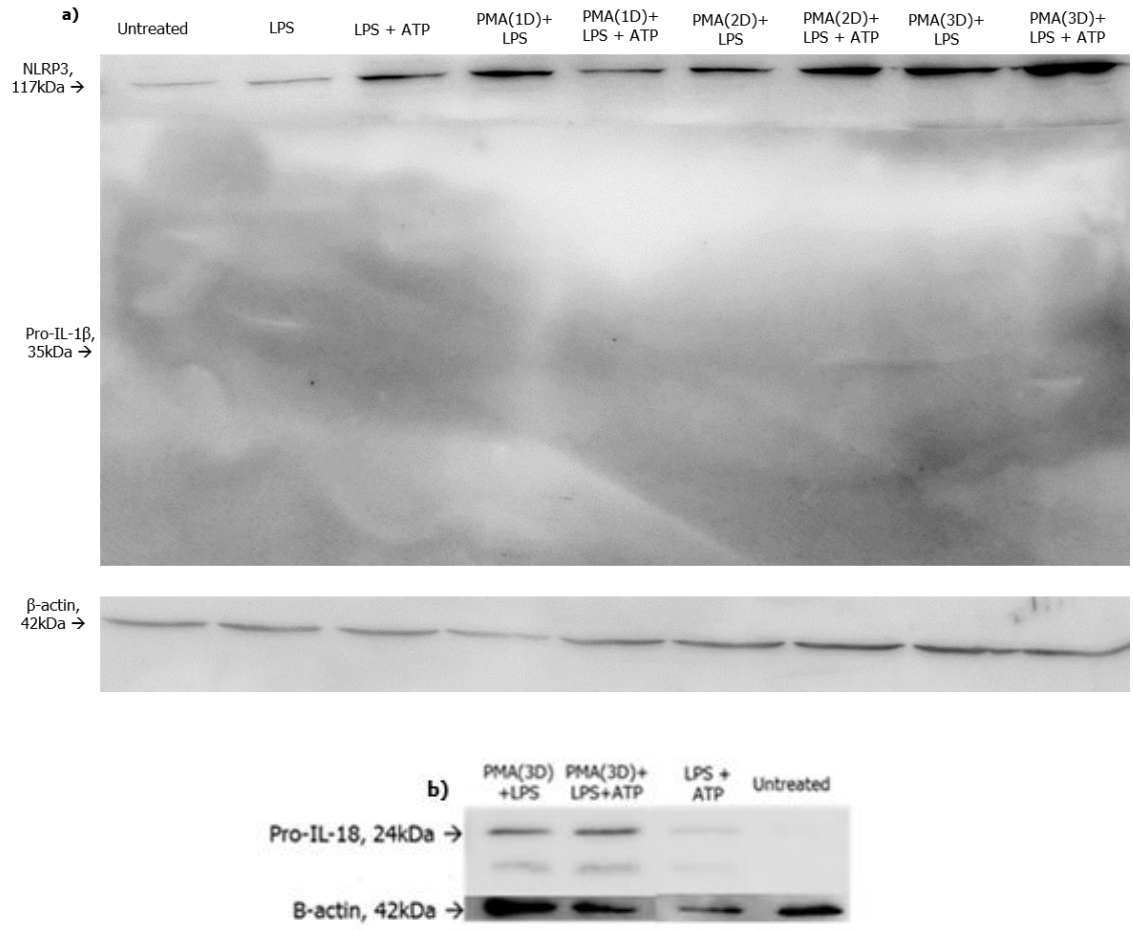

Figure 3-8 Signal 1 and Signal 2 expression in HUVEC.

Western blot investigating the expression of Signal 1; NLRP3 (**a**) and pro-IL-18 (**b**) in 50µg HUVEC lysates. PMA was used for 1, 2 and 3 days followed by LPS 5µg/ml (for Signal 1 activation) ± bzATP 300 µM for Signal 2 activation. Expression of pro-IL-1β was not observed.

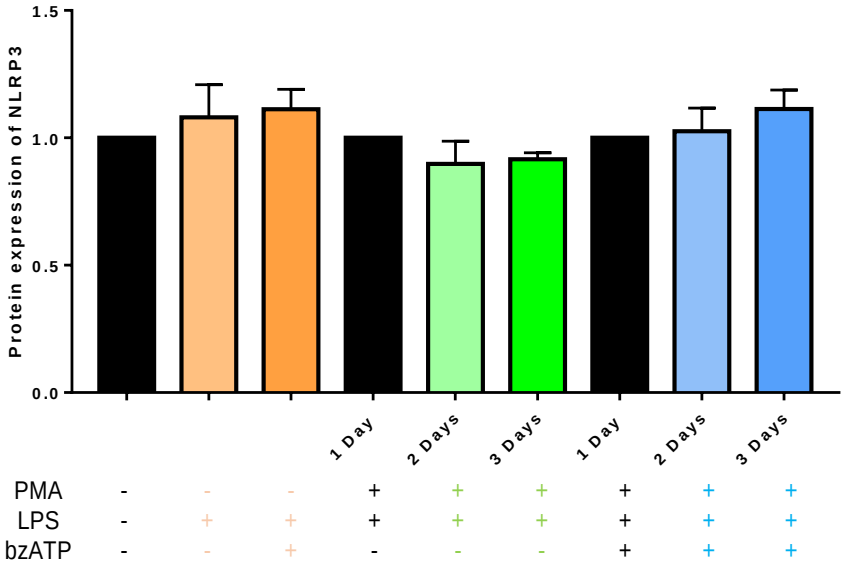

Figure 3-9 Quantitation of Signal 1 expression in HUVEC.

Quantitative analysis of Western blots from **Figure 3-8** showing the expression of Signal 1; NLRP3 only, relative to β-actin. PMA was used for 1, 2 and 3 days to activate cells followed by LPS 5µg/ml (for Signal 1 activation). There was no obvious trend with increasing time of exposure to PMA. Data presented as mean ± SEM, n=3, relative to β-actin. Statistical analysis (ANOVA) was used to compare cells treated for 2 and 3 days versus 1 day treatment.

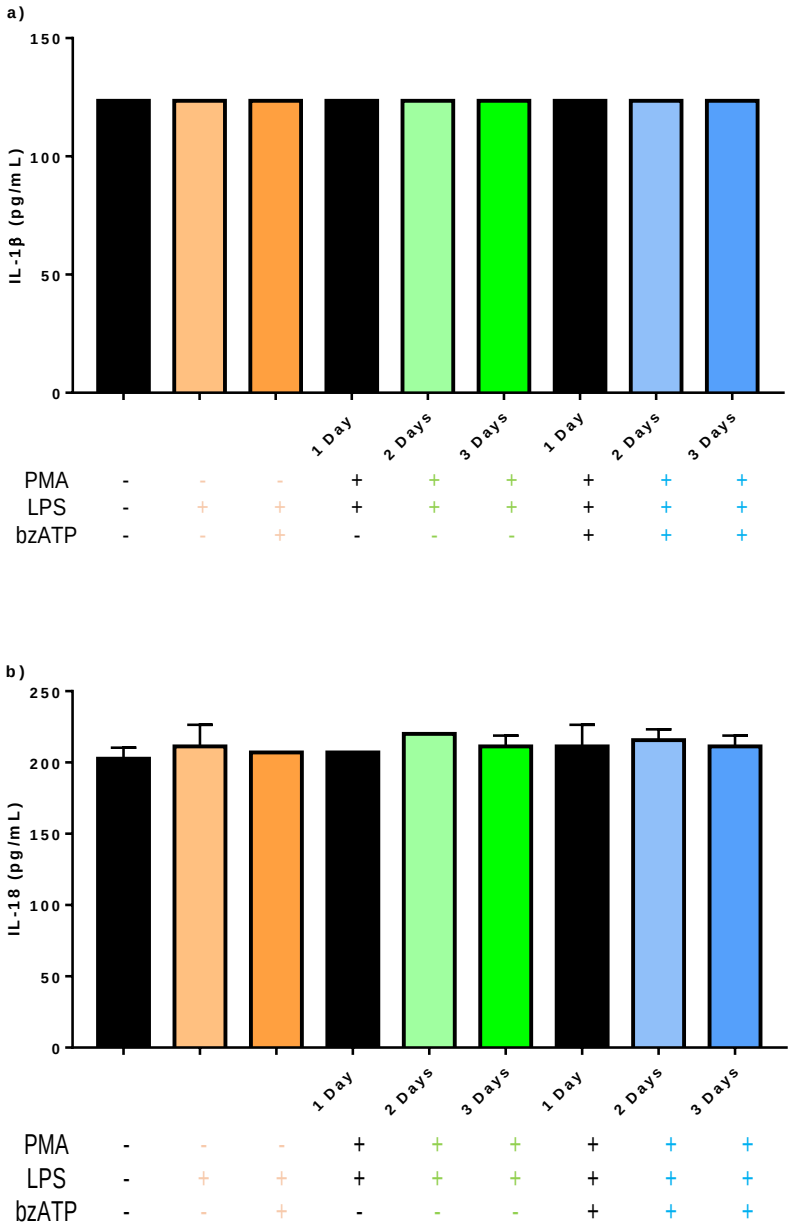

Figure 3-10 Signal 2 expression in cell culture supernatants of HUVEC.

Media from experiments shown in **Figure 3-8** was analysed for active IL-1β (**a**) and IL-18 (**b**) release by ELISA. Background levels of IL-1β and IL-18 were observed for all samples. Data presented as mean ± SEM, n=3. Statistical analysis (ANOVA) was used to compare cells treated for 2 and 3 days versus 1 day treatment.

3.4.4 NLRP3 inflammasome priming and activation pathways in EA.hy926 cells

EA.hy926 cells are a cell hybrid fusion of HUVEC and a human cancer cell line (Ahn et al., 1995). Their use in cardiovascular biology is well reported (Russell et al., 2000). NLRP3 inflammasome Signal 1 and Signal 2 were therefore investigated in this relevant model. PMA (5ng/ml) was used for 1, 2 and 3 days as with THP-1 cells followed by LPS (5µg/ml) for Signal 1 activation ± bzATP (300 µM) for Signal 2 activation. In addition, EA.hy926 cells were also treated without PMA.

Western blot and quantitative densitometry values of Western blots of lysate for untreated and all treated EA.hy926 cells are represented in **Figure 3-11 and Figure 3-12** Three antibodies were used on these gels; anti-NLRP3, anti-IL-1β and anti-IL-18. Results showed the presence of Signal 1 (NLRP3, pro-IL-1β and pro-IL-18) in all treated EA.hy926 cells. However, less expression of NLRP3 was noted in EA.hy926 cells without PMA treatment as observed for HUVEC (by 4 fold) (**Figure 3-12a**). Interestingly, pro-IL-1β expression was only observed when PMA was used suggesting perhaps PKC activation is required for Signal 1 in EA.hy926 cells (**Figure 3-12b**). There was no obvious difference in expression when comparing different PMA treatment times.

Signal 2 activation (assessed as release of active IL-1β and active IL-18 into cell media) by EA.hy926 cells was measured by Magnetic Luminex Assay ELISA (**Figure 3-13**). Untreated and treated EA.hy926 cells were analysed in 3 repeats. Active IL-1β secretion was slightly lower than for HUVEC cultures. Following PMA treatment, LPS increased synthesis and/ or release of IL-1β, between 4 to 5 fold (**Figure 3-13a**). There was a significant reduction of IL-1β release after 3 days treatment with PMA, compared to 1 day treatment suggesting prolonged exposure to PMA downregulated the inflammasome. Interestingly, bzATP did not affect IL-1β levels produced by these cells (**Figure 3-13a**). The secretion of active IL-18 was similar in all treated and untreated EA.hy926 cell media samples, indicating background levels of IL-18 (**Figure 3-13b**).

Subsequently, the cell lysates of untreated and treated EA.hy926 cells were analysed for HMGB1 expression by Western blot (**Figure 3-14**). Previous studies showed that many cell types express HMGB1 as a pro-inflammatory cytokine (Lotze and Tracey, 2005) which might be useful in investigating other inflammasome pathways in EA.hy926 cells. However, results demonstrated that PMA and LPS did not significantly increase the HMGB1 expression. Further treatment with bzATP administration also did not show any significant effect on HMGB1 expression.

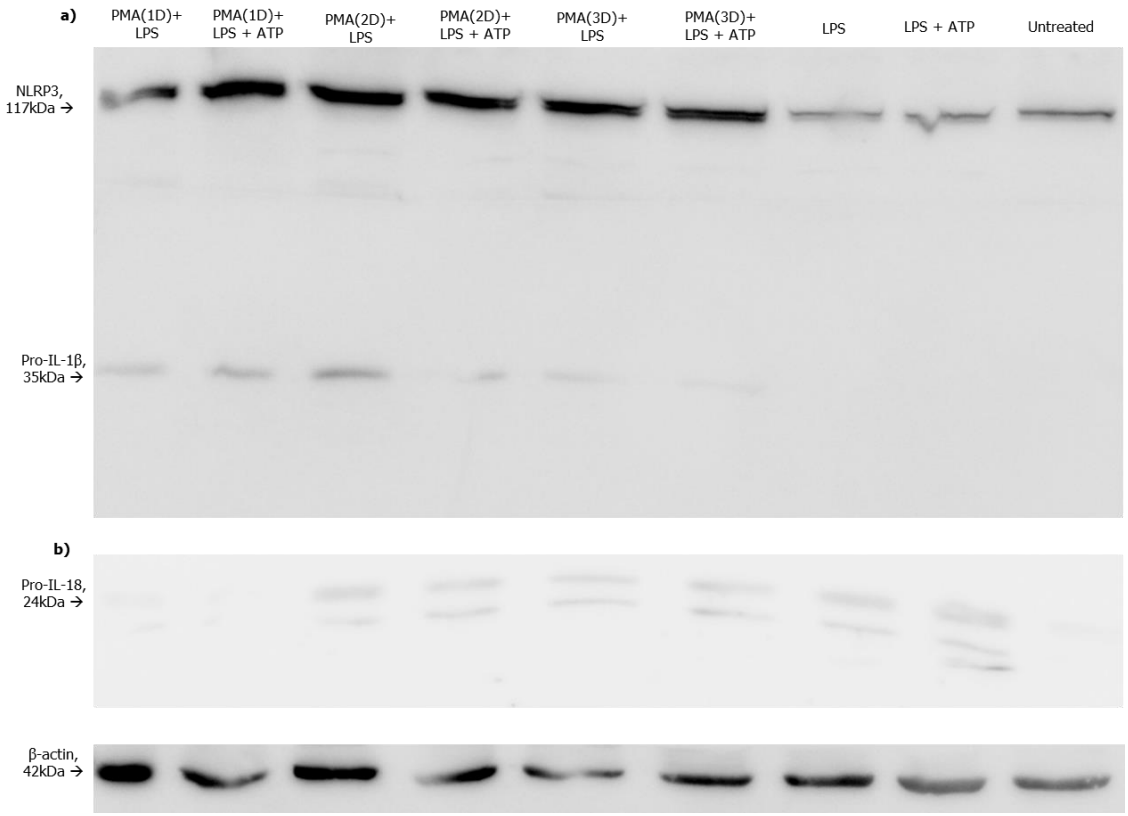

Figure 3-11 Signal 1 and Signal 2 expression in EA.hy926 cells.

Western blot showing the expression of NLRP3 and pro-IL-1β (Signal 1) in treated EA.hy926 cells (**a**). Cells were treated with PMA for 1, 2 and 3 days followed by LPS (5µg/ml) (for Signal 1 activation) ± bzATP (300 µM) for Signal 2 activation. Pro-IL-18 band was visualised following stripping of the immunoblot (**b**).

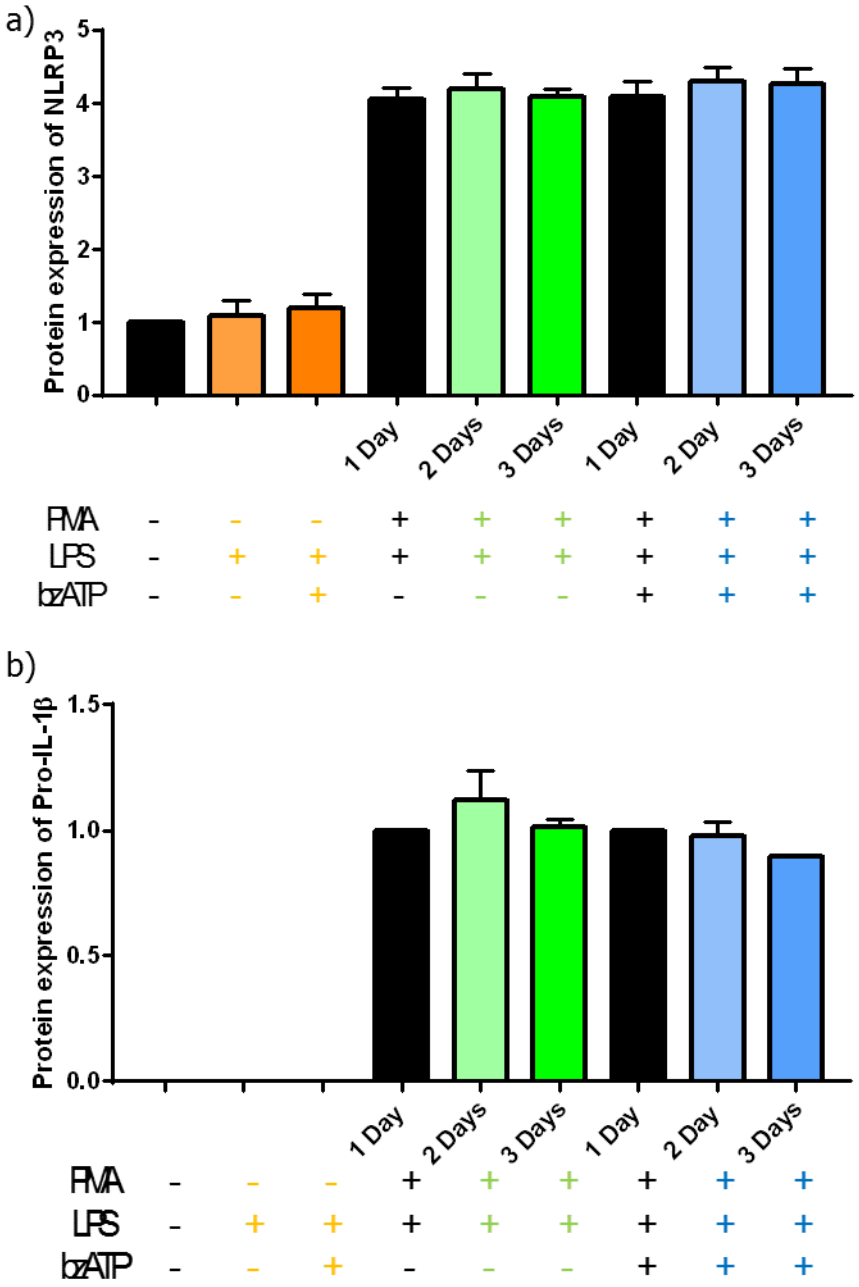

Figure 3-12 Quantitation of Signal 1 expression in EA.hy926 cells.

Quantitative analysis of Western blots from **Figure 3-11** showing the expression of Signal 1; NLRP3 (**a**) and pro-IL-1β (**b**), relative to β-actin. PMA was used for 1, 2 and 3 days to activate NF-κB followed by LPS (5μg/ml) (for Signal 1 activation). There were no significant differences in NLRP3 and pro-IL-1β expression for either 1, 2 or 3 days of PMA treatment with or without bzATP. Data presented as mean ± SEM, n=3, relative to β-actin. Statistical analysis (ANOVA) was used to compare cells treated for 2 and 3 days versus 1 day treatment.

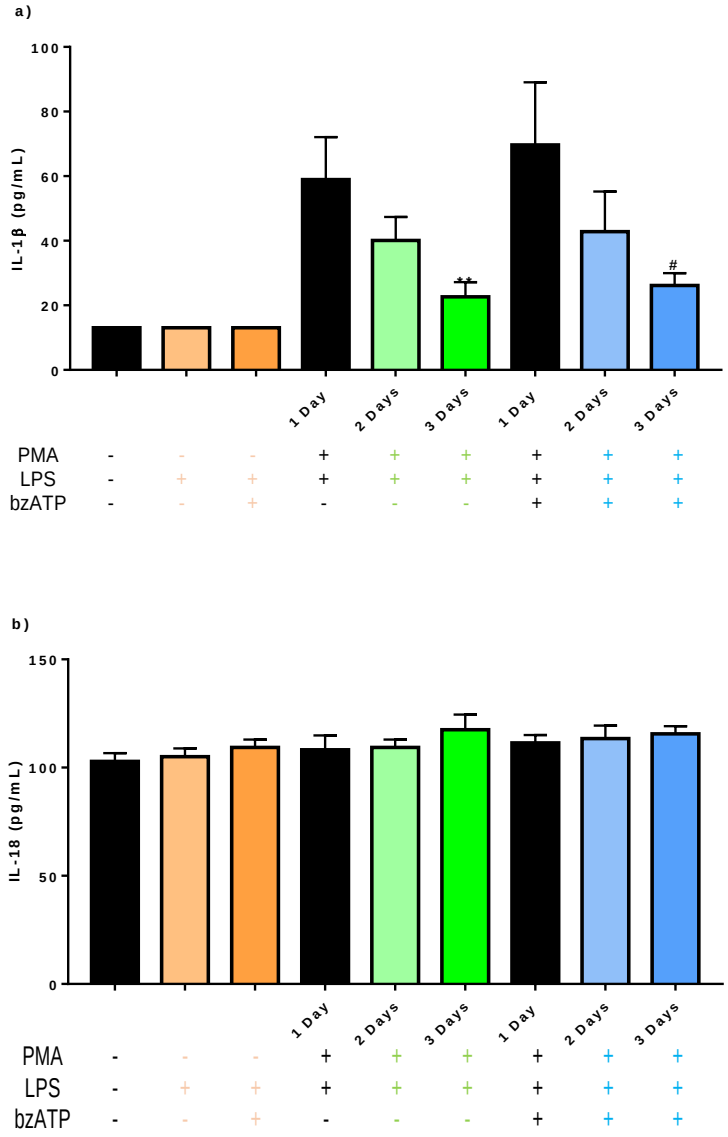

Figure 3-13 Signal 2 expression in cell culture supernatant of EA.hy926 cells.

Media from experiments shown in **Figure 3-11** was analysed for active IL-1β and IL-18 release by ELISA. Results showed increased IL-1β release upon treatment with PMA in the presence or absence of bzATP suggesting PMA/ LPS Signal 1 and activation of NLRP3 inflammasome in EA.hy926 cells. There was no evidence that bzATP acted as a potent Signal 2 activator in these cells. Statistical analysis showed that cells treated for 3 days with PMA produced less IL-1β compared to 1 day treatment, with or without bzATP exposure (**a**). Data are presented as mean ± SEM, n=3 (**$p<0.01$ and $^{\#}p<0.05$ as compared to 1 day treatment, respectively). IL-18 release was not obviously changed for any of the treatments (**b**). Statistical analysis (ANOVA) was used to compare cells treated for 2 and 3 days versus 1 day treatment.

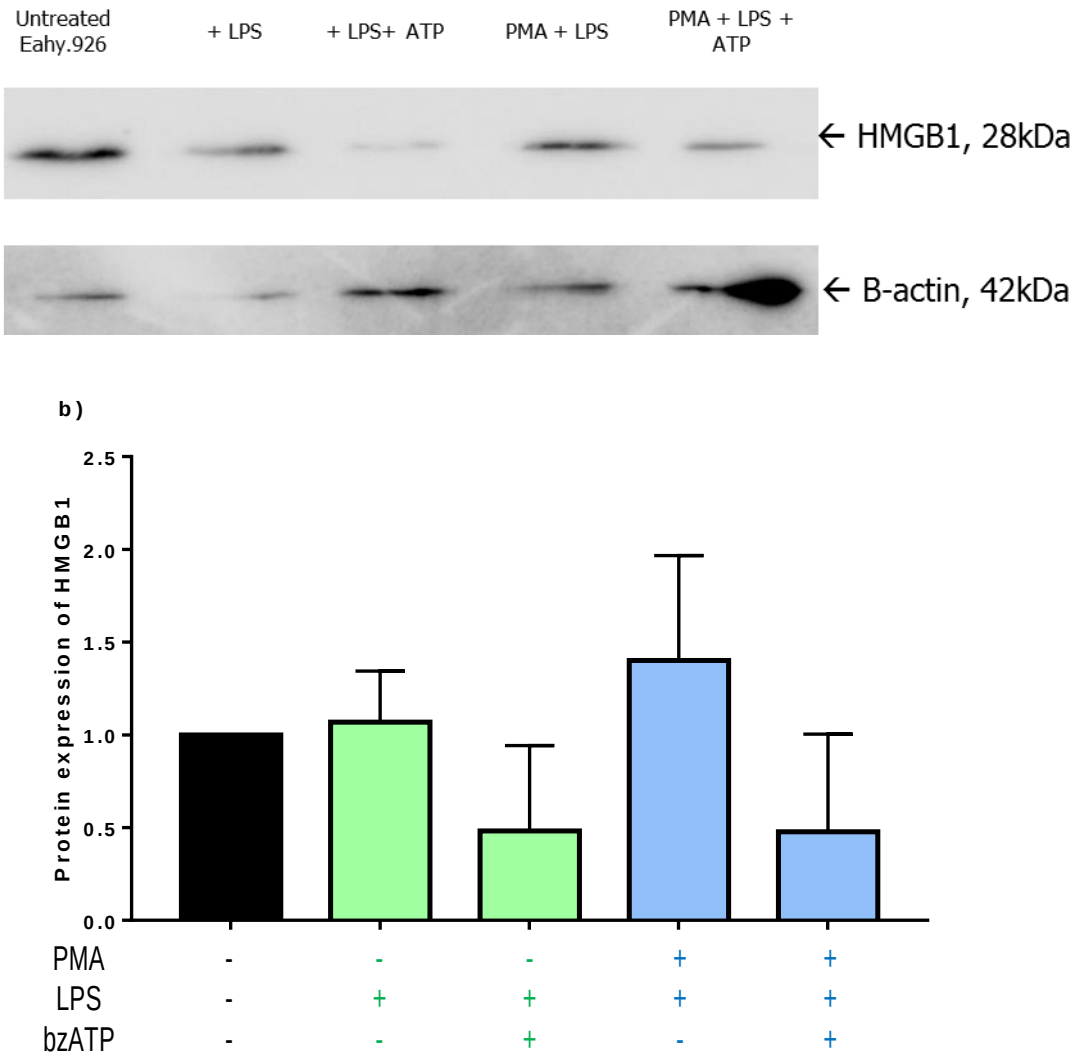

Figure 3-14 HMGB1 expression in EA.hy926 cells.

Cell lysates of untreated and treated EA.hy926 cells were investigated for HMGB1 (**a**). LPS 0.1µg/ml was added with or without bzATP for Signal 1 activation. PMA was used for 1 day only to activate NF-κB followed by LPS, with or without bzATP. Statistical analysis (ANOVA) were used to compare treated cells to untreated EA.hy926 cell of relative HMGB1 expression to β-actin (**b**). Data are presented as mean ± SEM, n=3 by ANOVA, relative to β-actin expression.

3.5 Discussion

3.5.1 Signal 1 activation in THP-1 cells

THP-1 cells resemble human primary monocytes and can be differentiated to macrophages using certain stimuli, for example, phorbol myristate acetate (PMA). PMA at 5ng/ml also activates NF-kB (Park et al., 2007), hence triggering Signal 1 of the NLRP3 inflammasome. In initial experiments, 5µg/ml LPS was used to trigger Signal 1. Surprisingly, LPS-treated, PMA differentiated, THP-1 cells showed both Signal 1 and Signal 2 activation even without bzATP administration. This might be due to effect of PMA that increases CD14 expression which acts as pattern recognition receptor especially for endotoxin LPS (Jersmann, 2005). Furthermore, activation of caspase-1 (canonical pathway) or caspase-5 (non-canonical pathway) might be triggered by LPS via Pycard or the C-terminal segment of NALP1 hence cleaving pro-IL-1β to the active form. However, the precise molecular mechanism is still unknown (Martinon et al., 2002).

Several concentrations of LPS also were tested after the PMA treatment. A high concentration of LPS was noticeably causing cell death (Wright et al., 1990). However, all concentrations of LPS showed almost similar Signal 1 expression.

When using low concentrations of LPS, no obvious differences in inflammasome activation were seen regardless of treating with PMA for 1 to 3 days. The concentration of PMA used was within the recommended range mentioned in previous studies (Schwende et al., 1996b). Additionally, this range of PMA concentration is sufficient to generate THP-1 cell differentiation without any unwanted gene upregulation, hence masking the effect by subsequent agents (Park et al., 2007). So it was decided to treat cells for one day with PMA.

ASC speck formation, detected by immunofluorescence was also used to confirm Signal 1 activation in treated, differentiated THP-1 cells. Treatment with LPS significantly triggered ASC activation, hence forming ASC-speck like bodies within cells suggesting NLRP3 inflammasome Signal 1. Subsequent treatment with bzATP causes Signal 2 activation hence following the formation and release

of IL-1β and IL-18 to the cell media, the inflammasome is down regulated and ASC specks disappear via apoptosis (Kumar et al., 2013).

As a model of professional immune cells, monocytic THP-1 cells manifest mature macrophage features such as adherence to the flask and enlarged nuclei upon being activated by PMA (Daigneault et al., 2010). Furthermore, PKC is believed to activate NF-κB via protein phosphorylation (Baeuerle and Henkel, 1994) (**Figure 3-15** and **Figure 3-16**). PMA or 1,25-dihydroxyvitamin D_3 (VD3) are well known as stimulators of monocyte-macrophage differentiation. However, studies show that activation via VD3 leads to incomplete macrophage differentiation (Auwerx, 1991). Because of this, PMA was used for this study.

In this study, predictably, THP-1 cells showed marked morphological changes consistent with differentiation into activated macrophages. PMA was used initially for 1 to 3 days for macrophage differentiation followed by LPS administration for Signal 1 activation (Martinon et al., 2002). As expected, no expression of either NLRP3 or pro-IL-1β was observed in untreated THP-1 cells. Furthermore, the production of NF-κB from differentiated THP-1 cells remains constant (Takashiba et al., 1999).

Compared with exposure for 1 day, treatment with PMA for 2 or 3 days did not showed any significant increase in the production of NLRP3 or pro-IL-1β after LPS administration. Presumably 1 day was sufficient for effective macrophage maturation (Lund et al., 2016). After the administration of LPS as a PAMP agent and activation of transcription factor, NF-κB translocation from the cytoplasm into the nucleus, NLRP3 transcription and translation are activated which is associated with higher levels of TNF-α secretion (Takashiba et al., 1999).

In this study, extracellular bzATP (adenosine triphosphate) was used simply to activate Signal 2. However, bzATP treatment revealed Signal 1 activation as well. BzATP mediates K^+ efflux extracellularly through plasma membrane channels, specifically highly polymorphic cation-selective channel, $P2X_7$ receptor (Cicko et al., 2015). This channel produces pore formation on the plasma membrane which stays open as long as it is bound to bzATP, which is essential for later active IL-1β release (Lister et al., 2007). In the present study, 2, 3-*O*-(4-benzoylbenzoyl)-

ATP (BzATP) was used rather than ATP itself in view of its 5 to 10 fold higher potency especially in investigating the $P2X_7$ receptor from other P2X receptor types. Hence, this provides more K^+ efflux and Ca^{2+} influx (Michel et al., 2001).

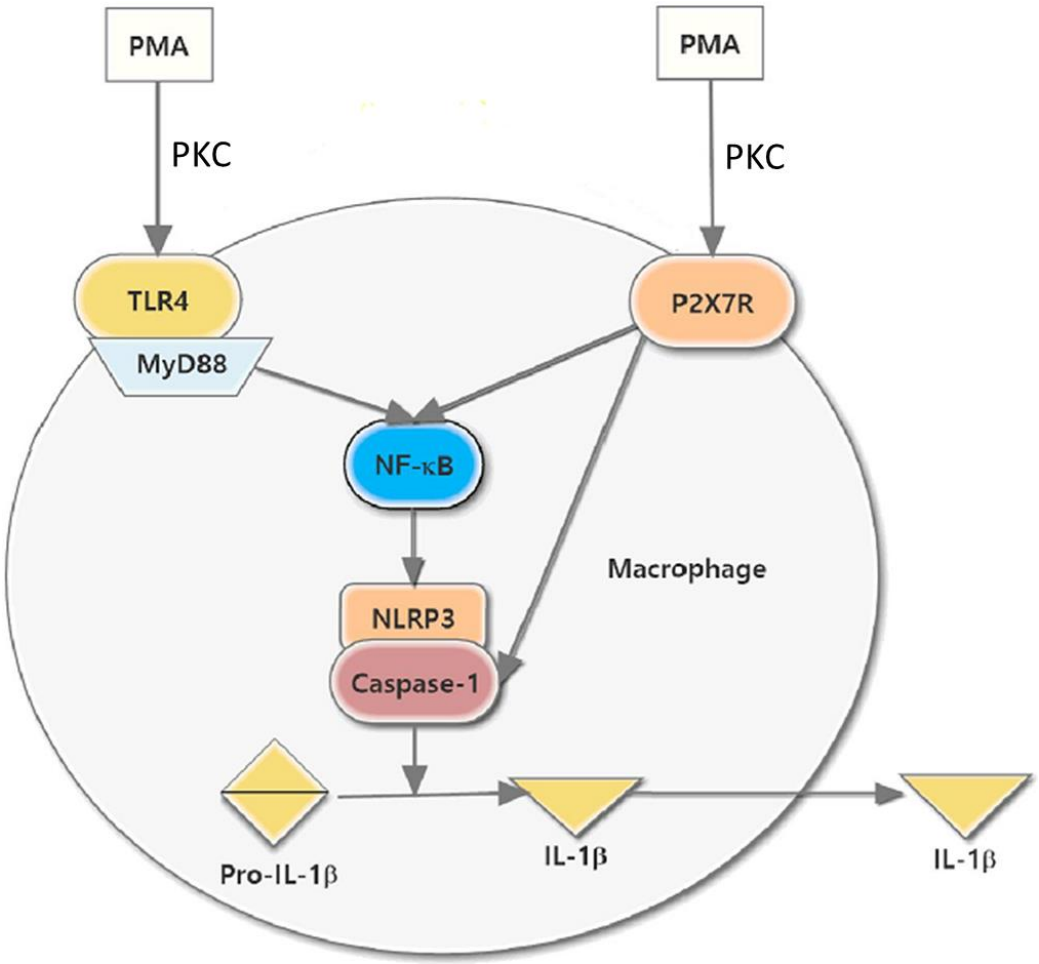

Figure 3-15 Schematic diagram shows Intracellular events by PMA leading to NF-κB activation.

PMA administration leads to upregulated $P2X_7R$ and TLR4 via MyD88 hence stimulates the active form of NF-κB. PKC plays a key role in the activation of NF-κB-dependent transcription. NF-κB plays an active role in cytoplasmic and nuclear signalling; NLRP3 inflammasome complex. Adapted from (Kong et al., 2016).

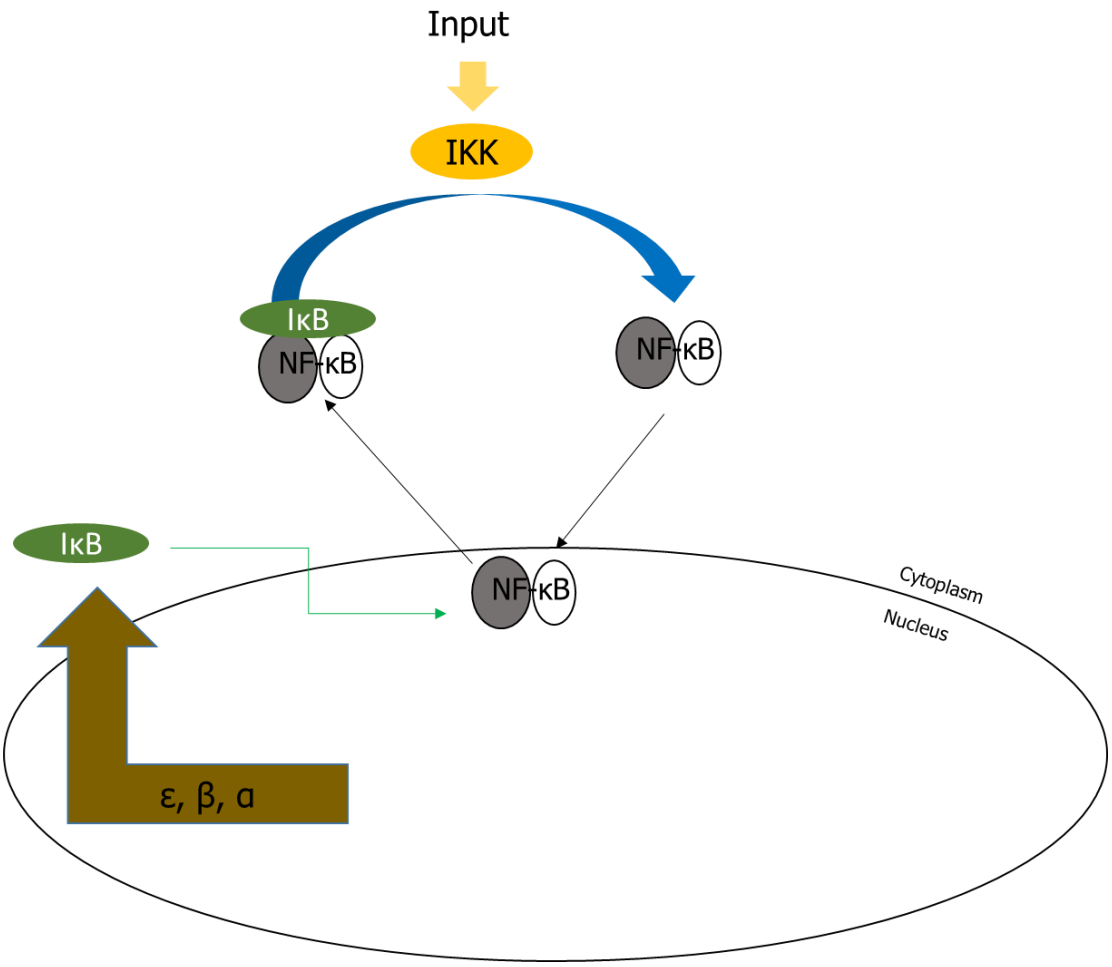

Figure 3-16 Basic IkB-NF-kB signalling model.

NF-kB is held inactive in the cytoplasm of non-stimulated cell by three IkB isoforms. During cell stimulation by PMA, IKK complex is activated, leading to phosphorylation and ubiquitination of the IkB proteins. Free NF-kB translocated to the nucleus, activating genes including NLRP3 inflammasome components. IkBα, IkBβ & IkBε are synthesised at steady rate, allowing for complex temporal control of NF-kB activation involving negative feedback. IKK: Inhibitor-kB kinase. Adapted from (Ihekwaba et al., 2004).

bzATP treatment did not increase Signal 1 expression in THP-1 cells in the presence of LPS, suggesting LPS was able to prime differentiated THP-1 cells optimally. It was explained previously that a second stimulus after LPS such as bzATP is required for Signal 2 NLRP3 activation (Bauernfeind et al., 2009).

Further study investigated the concentrations of LPS to induce the production of Signal 1 in THP-1 cells. Theoretically, LPS binds to LPS-binding protein (LBP) in plasma to form LPS-LBP complexes. These complexes are considered one of the potent triggers for macrophages to activate the NLRP3 inflammasome priming (Tobias et al., 1993). Previous study explained the relationship between LPS concentration with CD14 and MAPK for inflammatory response pathway by the activation of NF-κB which might be beneficial for the future study (Moore et al., 2000). Higher concentrations of LPS (5µg/ml) for 24 hours did not make any difference compared to lower concentrations (0.1µg/ml) in promoting Signal 1 expression of the inflammasome. Furthermore, it has been shown previously that the lower concentration of LPS (0.1µg/ml) is able to activate NF-κB to induce inflammasome Signal 1 (Zhang et al., 1999).

Furthermore, Signal 1 activation was confirmed by ASC speck formation. ASC is dormant inside the nucleus as well in the cytoplasm and is responsible for caspase-1 activation triggered by PAMPs or DAMPs (Stutz et al., 2013). Subsequently, ASC dimers or 'pyroptosomes' are formed rapidly to generate up to 10µm size ASC-speck like structures which can be visualized by fluorescence microscopy. However, not all LPS treated, differentiated THP-1 cells manifest this mechanism as ASC is unnecessary for NLRC4 inflammasome formation or involved in non-canonical pathways (Hara et al., 2013). However, less ASC-speck like formation was observed intracellularly after bzATP administration which agreed with previous studies demonstrating the release of ASC-specks extracellularly after ATP administration (Baroja-Mazo et al., 2014). In addition, studies also mentioned about the released of ASC-speck by dying cells leading to cleavage of extracellular pro-IL-1β, hence activating inflammasomes (Guo et al., 2015).

3.5.2 Signal 2 activation in THP-1 cells

As previously discussed, two signals are needed to activate the NLRP3 inflammasome complex; Signal 1 or priming and Signal 2 or activation. Signal 1 was measured by expression of NLRP3 and pro-IL-1β by Western blotting. Signal 2 was indicated by cleaved IL-1β in Western blot or more reliably, released extracellularly and measured quantitatively by an ELISA method. Studies showed various agents are able to activate the Signal 2 inflammasome such as ATP, cholesterol crystals, silica, asbestos, nigericin and uric acid (Leemans et al., 2011).

bzATP has been shown to induce the release of IL-1β from LPS-primed THP-1 cells (Grahames et al., 1999). In the current study, extracellular bzATP as a DAMP was used in view of its high affinity for the $P2X_7$ receptor on the plasma membrane which results in pore formation (Rayah et al., 2012). This lowers the level of K^+ intracellularly by the process of K^+ efflux as well as Ca^{2+} influx. Furthermore, it is well known that K^+ efflux triggers caspase-1 activation by the process of proteolysis hence allowing subsequent conversion of the pro-IL-1β to active IL-1β.

Furthermore, both IL-1β and IL-18 were increased suggesting bzATP promotes Signal 2 activation by stimulating caspase-1 cleavage and hence activates the robust production of mature IL-1β and IL-18 (Mariathasan et al., 2006). Further investigation of TNF-α might be helpful to study the relationship of bzATP with NLRP3 inflammasome pathways especially promoting caspase-1 cleavage (Franchi et al., 2009).

The mechanism of NLRP3 inflammasome activation by bzATP may still be questionable in this study in view of role of Ca^{2+} released from endoplasmic reticulum during stress by bzATP via C/EPB homologous protein (CHOP) (Murakami et al., 2012). Hence, further investigation using a Ca^{2+} blocker might helpful to study the main effect of bzATP in THP-1 cells.

ELISA showed no active IL-1β and active IL-18 were produced in untreated THP-1 cells, as expected. These data are in line with the biological state of this human

monocytic cell line which is not responsive if no DAMP or PAMP are introduced, hence it remains dormant (Auwerx, 1991). Extensive investigations were not performed with THP-1 cells treated with LPS alone since studies showed that LPS alone does not stimulate IL-1β release (Martinon et al., 2004).

The level of IL-1β release was higher than IL-18 release (15 fold) perhaps due to different mechanisms of caspase-1 activation. One study revealed that active IL-18 formation may not necessarily be dependent on caspase-1 but instead relies on neutrophil proteinase 3 (PR3) enzyme (Sugawara et al., 2001). Additionally, bioactive IL-18 release is considered to be a more important cytokine in T-cells, NK cells and neutrophils (Omoto et al., 2010). Other than conventional IL-1β-converting enzyme (caspase-1) that cleaves pro-IL-18 to the mature form of IL-18 at the at L-Aspartic acid-L-Tyrosine peptide bond site, caspase-3 also plays a major role in this cleavage but at a different processing sites; L-Aspartic acid-Serine peptide and L-Aspartic acid-Asparagine peptide bond sites (Akita et al., 1997). Hence, further investigation of caspase-1 and caspase-3 dependent IL-1β and IL-18 cleavages might be helpful to clarify the different pathways of NLRP3 inflammasome activation in THP-1 cells; either canonical or non-canonical.

3.5.3 Signal 1 activation in endothelial cells

To date, there are a paucity of studies with regards to NLRP3 inflammasome activation in human endothelial cells. In this thesis attempts to activate NLRP3 inflammasome in endothelial cells followed the methods tested in THP-1 cells. In untreated HUVEC, there was minimal expression of Signal 1 and no evidence for Signal 2 expression at all, indicating the HUVEC cells do not form an active NLRP3 inflammasome complex. However, it is possible that the end products of the NLRP3 inflammasome are different in endothelial cells and are not IL-1β and IL-18. Additionally, HUVEC cells need PMA treatment to produce higher Signal 1 expression which supports the idea that PMA acts as an NF-kB activator for triggering Signal 1 (see **Figure 3-15**).

However, in EA.hy926 cells, even though the findings were similar in HUVEC cells, additionally EA.hy926 cells showed low levels of Signal 2 expression. This was considerably lower than that observed in THP-1 cells. This might be because this type of cell has a cancerous origin that has an ability to produce a strong inflammasome activation (Liang et al., 2017). However, further investigation of the full transcriptome might be needed to investigate gene expression in response to NLRP3 activation, especially for HUVEC.

Despite exhibiting similar endothelial phenotypes, there are some differences between HUVEC and EA.hy926 cells after exposure to certain stimuli (Kraemer et al., 2011). EA.hy926 cells exhibit an additional large number of genes, higher expression of thrombomodulin and protein C activation as compared to HUVEC. Certain pro-inflammatory markers such as IL-6 and IL-8 might be expressed differently in EA.hy926 and HUVEC following induction with LPS (Unger et al., 2002). Hence, HUVEC should be considered as a first endothelial cell line of choice which resembles primary cells *in vivo*.

Even though study about endothelial cells as a vessel barrier is well established, more investigations are now focused on its adaptation towards specific needs in terms of time and location. Recently, more active research is being carried out on endothelial cells, such as its effect on thrombosis, coagulation, growth factors, vasodilators, and inflammatory mediators as in our study (Galley and Webster,

2004). Hence, in the sections below, our current understanding of the relationship between the NLRP3 inflammasome and endothelial cell functions is reviewed especially the release of inflammatory mediators such as IL-1β and IL-18.

3.5.3.1 HUVEC

There are two main classifications of endothelial cells; microvascular endothelial cells and macrovascular. However, *in vitro* studies using microvascular endothelial cells are focused more on the development of blood vessels or angiogenesis (Jackson and Nguyen, 1997). Additionally, macrovascular endothelial cells such as HUVEC have different growth requirements such as extracellular components and mitogens that might cause differences in the production of inflammatory markers (Terramani et al., 2000).

The research about HUVEC has been well established for 40 years (Jaffe et al., 1973). HUVEC were isolated from fresh human umbilical cord by collagenase methods from the internal part of umbilical veins. Furthermore, the important relationship between this type of cell and inflammatory responses towards the production of cytokines has been well studied (Jirik et al., 1989). However, limitations have been identified in handling HUVEC cells, such as limited life span of primary cultured HUVEC and loss of some of properties or phenotype, for instance cellular memory, plasticity and multistability after early passages (Regan and Aird, 2012). Moreover, HUVEC might manifest different characteristics between donors or between batches (Bouïs et al., 2001).

In view of the limitations of HUVEC, the need for stability of primary endothelial cells supported the emergence of newly immortalized endothelial cells, EA.hy926 cells. This permanent type of cell line was established in 1983 via fusion of HUVEC with human lung carcinoma cell line A549 (Edgell et al., 1983, Lieber et al., 1976). These cells exhibit rapid growth, low senescence, minimized requirement of growth factors and upregulate certain immunoglobulins for inflammation signalling (Thornhill et al., 1993). Hence, they are suitable for some forms of endothelial cell research.

Treatment of both endothelial cell types with PMA was undertaken to mirror the methods used for THP-1 cells. However, no obvious morphology differences were shown in either endothelial cell type treated with PMA even though treatment was for up to 3 days. This agree with literature which showed that PMA was able to activate PKC in endothelial cells yet there were no obvious changes in morphology (Scarpati and Sadler, 1989).

Here, it was shown that NLRP3 protein was not expressed in untreated HUVEC. Surprisingly, this is amongst the first studies to demonstrate the expression of Signal 1 in HUVEC following LPS. The expression of inflammasome components in HUVEC after LPS treatment might be due to the presence of TLR on the membrane surface as reported previously (Yin et al., 2009b). Furthermore, treatment with this major cell wall component of gram negative bacteria is believed to activate NF-κB in HUVEC hence promoting Signal 1 activation (Talreja et al., 2004). However, further investigations of other pattern-recognition receptors are needed to study the relationship of LPS and HUVEC (Land, 2013).

Pro-IL-1β was not expressed at all in HUVEC either with LPS or in combination with PMA. This is not surprising as a previous study demonstrated that HUVEC only expressed IL-1ra (receptor antagonist) but not IL-1β and IL-1α (Dewberry et al., 2000). However, TGF-β (transforming growth factor beta) should be investigated in future studies which might induce IL-1β or other types of inflammatory cytokines in HUVEC (Pihusch et al., 2005). Additionally, function of PKC-β (protein kinase C beta type) might be further investigated in view of inducing the production of advanced glycation end products (AGEs) in HUVEC hence promoting the inflammation process (Xu et al., 2010).

3.5.3.2 EA.hy926 cells

As in HUVEC, EA.hy926 cells showed expression of Signal 1; NLRP3 as well as pro-IL-1β. Unpredictably, untreated EA.hy926 cells showed expression of NLRP3 protein. Since EA.hy926 cells are formed by fusion of HUVEC and lung carcinoma cells, this might be explained by the presence of the cancerous phenotype in this cell. However, treatment with LPS did not increase the expression of NLRP3 protein. Further investigation of TNF-α might be helpful as LPS triggers TNF-α production in endothelial cells (Strieter et al., 1989).

Further treatment with LPS in PMA treated EA.hy926 cells did not show any differences in the expression of NLRP3 protein in Western blot either for 1, 2 or 3 days of PMA treatment, with or without bzATP. Even though there is not much literature investigating the effect of PMA on EA.hy926 cells, PMA is believed to activate apoptosis induction in addition to NF-κB and TNF-α activation (Rödel et al., 2012). TLR upregulation also has been identified by EA.hy926 activated by PMA hence triggering Signal 1 activation (Askari et al., 2014). Further investigations might be needed to assess the release of EPCR (endothelial cell protein c receptor) in PMA treated EA.hy926 cells in promoting inflammation (Xu et al., 2000).

Like HUVEC, untreated EA.hy926 cells also did not express pro-IL-1β. Furthermore, LPS treated EA.hy926 cells also did not show any pro-IL-1β expression. Even though a study showed that EA.hy926 cells treated with LPS showed reduced endothelial permeability and triggered inflammation, it does not necessarily express pro-IL-1β (Kaneider et al., 2007). Further investigation of other pro-inflammatory cytokines and the non-canonical pathway of the NLRP3 inflammasome activation might be needed to rule out Signal 1 activation in EA.hy926 cells.

Similar to NLRP3 protein expression for Signal 1, EA.hy926 cells also showed obvious expression of pro-IL-1β upon administration of PMA in LPS treated EA.hy926 cells. It was shown previously that endothelial cell protein c receptor (EPCR) which is located on EA.hy926 cell surface might contribute to the release

of pro-inflammatory cytokines in PMA treated EA.hy926 cells (Villegas-Mendez et al., 2007).

Both HUVEC and EA.hy926 cells did not show any obvious differences in expression of either NLRP3 or pro-IL-1β for 1,2 or 3 days of PMA administration. This is supported by previous studies demonstrating that 24 hours treatment with PMA is an optimal duration to promote inflammation in endothelial cells, especially in triggering eNOS production and NO release by PKC downregulation (Schmitt et al., 2010). Hence, further investigation of nitric oxide synthase might be helpful to investigate pro-inflammatory mechanism in EA.hy926 cells following PMA (Fleming et al., 2001).

3.5.4 Signal 2 activation in endothelial cells

3.5.4.1 HUVEC

Similar to THP-1 cells, the end products of Signal 2 expression of both HUVEC and EA.hy926 cells were measured by ELISA following PMA, LPS and bzATP activation. However, no increased values of IL-1β and IL-18 were observed in treated HUVEC as compared to untreated HUVEC. Little work has been reported in the literature on this sort of protocol but further investigations need to be carried out to activate NLRP3 inflammasome in HUVEC.

There is a possibility that a dose of LPS (5µg/ml) might be related to other TLR pathway of immunological response via MyD88, which is one of the IL-1 receptor signalling molecules that activates the inflammasome (Faure et al., 2000). In addition, specific studies have been reported about a synergistic effect of homocysteine on LPS-treated HUVEC with respect to triggering pyroptosis by caspase-1 activation (Xi et al., 2016). The recently published NLRP3 inflammasome end product, HMGB1 (high mobility group box 1) might also be an alternative end product of PAMP stimulation of endothelial cells (Lu et al., 2014).

As mentioned, there is a paucity of studies on the NLRP3 inflammasome in HUVEC. One study triggered mtROS and intracellular ROS in HUVEC by the administration of cadmium ($CdCl_2$), a toxic compound usually found in industrial waste (Chen et al., 2016). In this study, $CdCl_2$ was administered after the LPS treatment resulting in the expression of active IL-1β and active caspase-1 in cell lysates. It is also possible that cell death is an end product of NLRP3 activation in endothelial cells. However, similar to our results, this study showed limited expression of pro-IL-1β (Signal 1) in HUVEC. Furthermore, it did not mention release of active IL-1β into the cell culture supernatant.

Another study mentioned that the red meat substance, TMAO (trimethylamine N-oxide) possibly primed the TXNIP pathways (thioredoxin-interacting protein), releasing inflammatory cytokines and affecting endothelial function in HUVEC by ROS generation (Sun et al., 2016b). In these experiments, the adaptor component of NLRP3 inflammasome, ASC also was measured by

immunofluorescence and ELISA which might be useful for future study of THP-1 cells (Compan et al., 2015). Additionally, an active component of the pesticide tetracholorobenzoquinone might be useful as a positive control for studying the role of HUVEC mediated ROS production and mitochondria DNA damage in releasing active-IL-1β as well as in signal 1 generation (Xia et al., 2016).

3.5.4.2 EA.hy926

Treatment of EA.hy926 cells with PMA and LPS showed expression by IL-1β in ELISA. However, upon treatment with LPS-only, with or without bzATP, they did not show any obvious increment compared to untreated EA.hy926 cells. A previous study in lung epithelial cells showed increased expression of IL-8 rather than IL-1β after treatment with LPS (Ghazarian, 2014) which might be further evaluated. Further investigation of ICAM-1, VCAM-1 and chemokine (C-X3-C motif) ligand 1 (CX3CL1) in LPS-induced endothelial cells should be carried out in EA.hy926 cells (Fang et al., 2017).

Surprisingly, treatment with PMA before LPS markedly increased IL-1β expression as compared to LPS-only treatment. Previous studies showed that PMA acts to cause translocation of PKC_{α} and PKC_{ε} from the cytosol to the cell membrane of EA.hy926 cells (Li et al., 1998). The implication of this is that this might trigger the NLRP3 inflammasome. Furthermore, in this study, treatment with PMA for more than 24 hours significantly decreased the IL-1β expression in a time dependent manner which might be due to the down-regulation of both PKC isoforms. Besides, treatment with PMA less than 24 hours is not recommended as previous study showed that the production of PKC would be reduced by the process of human cationic amino acid transporter inhibition (Gräf et al., 2001).

Further treatment with bzATP slightly increased the production of IL-1β (Signal 2 NLRP3 inflammasome) within 1 to 3 days of PMA treatment, respectively. As mentioned before, extracellular bzATP may activate $P2X_7$ receptor which modulates the NLRP3 inflammasome by autocrine or paracrine mechanisms (Abbracchio et al., 2006). Additionally, bzATP activates mitochondrial-derived ROS, hence triggering AMPK (AMP-activated protein kinase) leading to autophagy and further NLRP3 inflammasome production in EA.hy926 cells

(Maiuri et al., 2013). However, further study about non-canonical pathways of NLRP3 inflammasome (Caspase 4,5 and 11) activated gasdermin D protein in EA.hy926 cells should be carried out to rule out bzATP dependent inflammasome activation (Liu et al., 2016b).

No active IL-18 expression was observed in all treated EA.hy926 cells as compared to untreated EA.hy926 cells. Similar to HUVEC, there are limited studies of IL-18 production in EA.hy926 cells. One study mentions about Fas receptor (first apoptosis signal receptor) or CD95 triggering IL-18 and IL-1β production by the activation of caspase-8, which is present in the non-canonical pathway of NLRP3 inflammasome activation (Bossaller et al., 2012).

Further investigation of the alternative inflammasome end product, HMGB1 was performed in EA.hy926 cells. HMGB1 is non-histone chromatin-binding protein which plays a major role in acute inflammation, tumour metastasis and as an intracellular DNA transcription regulator (Sha et al., 2008). However, the effect of HMGB1 in endothelial cells is still less well described.

Following treatment of EA.hy926 cells with LPS and a combination of PMA and LPS, there was a trend for increased expression of HMGB1. A previous study suggested the receptor for advanced glycation end products, RAGE, is expressed in endothelial cells and interacts with released HMGB1 (Fiuza et al., 2003). Besides that, the production of HMGB1 also can be triggered by TLR2 and TLR4 activation (Park et al., 2004).

Interestingly, additional PMA treatment prior to LPS administration activates PKC transmigration into the nucleus and hence enhances HMGB1 expression (Oh et al., 2009). However, no significant expression of HMGB1 was observed in both LPS and PMA-LPS treatment which is best explained by the need for longer duration of LPS to activate p38 MAPK and NF-κB signal pathways (Wu et al., 2012). Furthermore, the release of HMGB1 protein could be delayed (Lu et al., 2013).

However, there was a trend (not statistically significant) for less expression of HMGB1 intracellularly after bzATP administration. This potent Signal 2 NLRP3

inflammasome activator might release the HMGB1 extracellularly by robust double-stranded RNA-dependent protein kinase (PKR) auto phosphorylation hence inducing pyroptosis (Lu et al., 2012).

3.6 Conclusions

In conclusion, NLRP3 protein expression was upregulated in THP-1 cells and active IL-1β and IL-18 were secreted into the media, suggesting the presence of both Signal 1 and Signal 2 of the NLRP3 inflammasome in this human macrophage cell model.

Both endothelial cell types (HUVEC and EA.hy926) showed evidence of low level Signal 1 activation. Despite HUVEC cells showing priming of the NLRP3 inflammasome, there was no evidence for either active IL-1β or active IL-18 synthesis. Surprisingly, EA.hy926 cells showed low levels of active IL-1β, when exposed to PMA, suggesting presence of an active NLRP3 inflammasome. This effect was down-regulated when the duration of exposure to PMA was increased for 24 to 48 to 72 hours. Importantly, other endpoints of inflammasome activation might be also significant and further work is required to investigate the mechanisms and significance of NLRP3 inflammasome activation for endothelial cell biology.

Chapter 4 To investigate the effects of intracellular generation of superoxide on the THP-1 cell NLRP3 inflammasome

4.1 Background

4.1.1 Mitochondria and the NLRP3 inflammasome

Mitochondria act as a powerful energy supply to cells by the process called oxidative phosphorylation to produce ATP (Frey and Mannella, 2000) but are also involved in apoptosis and signal translation and transduction (West, 2017). Furthermore, mitochondria regulate oxygen homeostasis in all eukaryotic cells which is important for survival (Brunelle et al., 2005). The basic understanding of mitochondrial functions has been studied intensively; however there are limited studies on the role of mitochondrial function and NLRP3 inflammasome activation.

Previous study showed that mitochondria play a major role in the production of innate immune responses by the receptor called RLR (retinoic acid-inducible gene–I-like receptor) especially by PAMPS (Iwasaki and Medzhitov, 2010). Subsequently, RLR, as an adaptor molecule, assembles with mitochondria antiviral signalling protein (MAVS) to activate NF-κβ as well as interferon regulatory factors (IRFs) hence leading to activation of T and B-cells (West et al., 2006).

Additionally, in the innate immune system, ROS production is controlled by the NADPH-oxidase-dependent-respiratory burst. This phagocytic response is needed for the lysis of intracellular microbes (West et al., 2011). Furthermore, in mammalian cells, the balance of oxygen haemostasis for cell survival is actively sensed by mitochondrial-generated reactive oxygen species. Hence, in certain hypoxia pathogenesis, mitochondrial respiratory chain complex regulates cell physiology through stabilization of protein hypoxia-inducible factor 1α (HIF-1α) (Brunelle et al., 2005).

The endoplasmic reticulum-mitochondria encounter structure (ERMES) creates the protein junction between mitochondria and the endoplasmic reticulum. This complex acts mainly for ion transport for maintaining mitochondrial morphology (Xue et al., 2017). Importantly, NLRP3 protein inside the endoplasmic reticulum senses the dysregulation of the mitochondria *via* accumulation of damaged, ROS-generating mitochondria , hence activating the adaptor or ASC, inside the perinuclear space where NLRP3-ASC co-localize with endoplasmic reticulum and mitochondrial organelles (Zhou et al., 2011).

The important sources of cellular ROS are the mitochondria as well as NADPH oxidases (NOXs). Initial studies on immune-related ROS focused on phagocyte NOX (Dunn et al., 2015). However, increasing evidence indicates that ROS production in the mitochondria, once thought to be an unwanted by-product of oxidative metabolism, is regulated by immune signalling pathways and is involved in immune responses (Dromparis and Michelakis, 2013). Mitochondria and mitochondrial ROS are also components of apoptosis, stem cell differentiation, autophagy and cellular or tissue level of inflammation. Therefore, the mitochondria provide a plausible link between inflammation and the metabolic state of the cell (Bauernfeind et al., 2011).

Physiological stress and energy deficit can trigger autophagy resulting in degradation of the cell as well as recycling the core metabolic activities (Wang et al., 2012). Autophagy can be induced by ROS produced by mitochondria and phagocyte NADPH oxidase (NOX2) in response to nutrient starvation and bacterial infection. Bacteria such as *Mycobacterium tuberculosis* inhibit autophagy by decreasing mitochondrial and NOX2-generated ROS. Autophagy, mitochondrial ROS, and NOX2-generated ROS have also been found to be important for extracellular trap formation (Dunn et al., 2015).

Mitochondrial ROS production triggered by NLRP3 activators is limited by autophagy clearance of defective mitochondria. ROS production leads to the relocation of NLRP3 to mitochondria-associated membranes (MAMs), where ASC is recruited thereby promoting NLRP3 inflammasome activation (Sorbara and Girardin, 2011). In addition, the NLRP3 inflammasome would promote the

activation of caspase-1 in the presence of Signal 1 leading to mitochondrial-dependent apoptotic cascade (Shimada et al., 2012). NLRP3 inflammasome activation depends upon the interplay between mitochondrial ROS generation, mtDNA release and regulation of mitophagy. It is important to determine whether mitochondrial ROS are definitely induced by NLRP3 activators, and elucidate the pathways leading to mitochondrial stress in each case (Zhou et al., 2011). Further evaluation of the relative contribution of mitochondria-triggered ROS and NADPH oxidase in NLRP3 inflammasome activation is required (Sorbara and Girardin, 2011).

In view of the observation that mitochondrial dysfunction drives NLRP3 inflammasome activation, it is believed that the NLRP3 inflammasome regulates cellular metabolism. Mitochondria generate huge amounts of chemical energy in the form of ATP from glucose, lipids, amino acids and nucleic acids with the assistance of oxygen. Instead of detecting each danger signal individually, the NLRP3 inflammasome monitors the activity of the mitochondrion, which acts as an integrator of danger signals, including those of metabolic origin. Excessive ROS production by mitochondria leads to activation of the inflammasome. It is known that many situations that lead to altered cellular metabolism can trigger the NLRP3 inflammasome. Other than PAMP, altered mitochondrial activity and cellular metabolism also leads to activation of the inflammasome by invading viruses or bacteria (Tschopp, 2011).

4.1.2 Generation of superoxide by paraquat (PQ) and mitoparaquat (mitoPQ)

Generally, superoxide and its products are considered biologically harmful and toxic to the living cells contributing to pathogenesis of many diseases including diabetes and reperfusion injury (Murphy, 2009). Excessive superoxide production, a by-product of cellular metabolism accumulates in the mitochondria hence promoting oxidative stress (Thannickal and Fanburg, 2000). Furthermore, ROS are able to damage lipids, DNA and proteins (Simon et al., 2000). However, there is a lack of understanding on the relationship between ROS damage of biomolecules and pathogenesis of disease.

PQ and mitoPQ are synthetic compounds (**Figure 4-1**) and produce superoxide by redox cycling; the process explained by repetitively coupled reduction and oxidation reactions, often involving oxygen and reactive oxygen species (Kappus, 1986). PQ is a non-selective herbicide, targeting cell apoptosis and necrosis by the production of ROS, hydrogen peroxide, hydroxyl radical, superoxide anions (Zhao et al., 2017a) as well as reducing ATP production which leads to NADPH (nicotinamide adenine dinucleotide phosphate hydrogen) biochemical disruption (Suntres, 2002).

PQ generates superoxide in universal cell compartments whereas mitoPQ produces superoxide in mitochondria (Hassan, 1984). Hence, it might need higher PQ concentration as compared to mitoPQ to produce the same mitochondrial effect. This is because mitoPQ consist of a lipophilic triphenylphosphonium (TPP) cation conjugated to PQ which specifically targets mitoPQ to mitochondria facilitated by the mitochondrial membrane potential (Robb et al., 2015).

Even though mitoPQ is well studied in developing mitochondrial superoxide in cell culture and isolated mitochondria, most of the literature also emphasized the relationship of PQ in apoptosis of cells mediated by cytochrome *c* release perhaps by the process of cardiolipin oxidation (Ott et al., 2007). This cytochrome *c* release also may be aggravated by increased calcium influx, hence weakening the electrostatic interaction between cardiolipin and cytochrome c, leading to robust ROS production (Ow et al., 2008).

Additionally, this pro-apoptosis factor (cytochrome *c*) together with Smac (Second mitochondria-derived activator of caspase) and AIF (apoptosis-inducing factor) further disrupted the membrane permeabilization of the mitochondria (Zamzami and Kroemer, 2001). The Dynamin related protein 1 (Drp1) is a protein that maintains dynamic reticular structure of mitochondria by the process called mitochondrial fission; this process is progressively disturbed by the present of PQ (Shenouda et al., 2011). Furthermore, oligomerization of Drp1 in response to

mitochondria oxidative stress lead to mitochondrial fragmentation and dysfunction (Ježek et al., 2018).

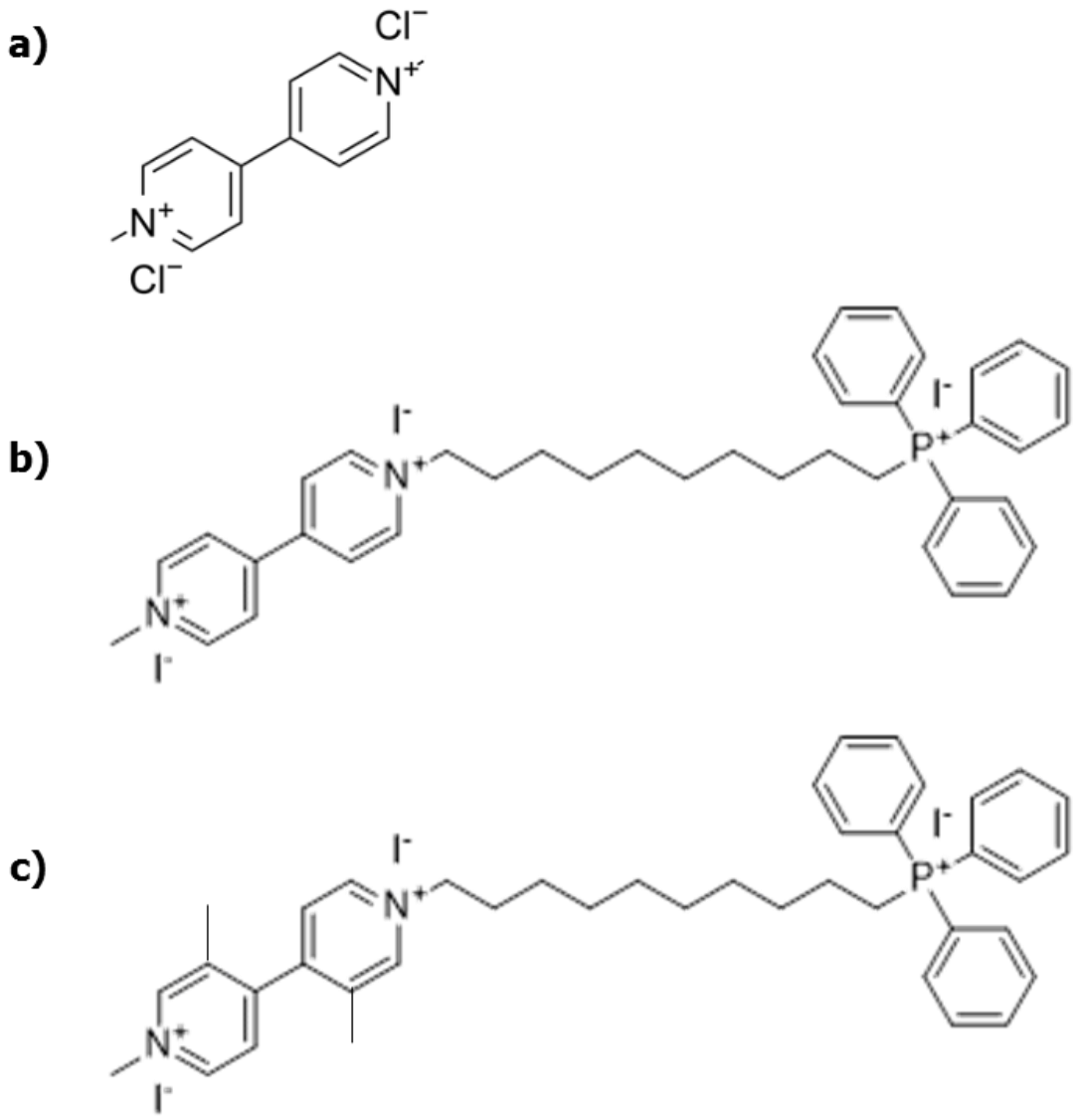

Figure 4-1 Chemical structure of paraquat (PQ), mitoparaquat (mitoPQ) and mitoParaquat (control) (mitoPQ(C)).

Schematic diagram shows (**a**) paraquat; 1,1'-dimethyl-4,4'-bipyridinium dichloride, $C_{12}H_{14}Cl_2N_2$, molecular weight 257.158, (**b**) mitoparaquat; triphenylphosphonium lipophilic cation conjugated to the redox cycler paraquat, $C_{39}H_{46}I_3N_2P$, molecular weight 954.48 and (**c**) mitoparaquat (control); $C_{41}H_{50}I_3N_2P$, molecular weight 982.55, which target mitochondria but does not generate superoxide anion.

Furthermore, PQ toxicity is predominantly assembled in pulmonary tissue, by the redox cycling mechanism that leads to intracellular oxidative stress. PQ accumulates up to ten times the concentration in plasma, via a polyamine uptake mechanism on the membrane of alveolar cells leading to pulmonary oedema and accumulation of fibroblast and collagen deposition (Dinis-Oliveira et al., 2008).

Certain antioxidants such as ascorbic acid have been shown to reduce paraquat toxicity effects (Hong et al., 2002). Additionally, manganese superoxide dismutase (SOD), glutathione peroxidase, catalase and cytochrome *c* oxidase are important antioxidant defences in mitochondria (Turrens, 2003). Mitochondrial uncoupling proteins such as UCP1 and UCP2 also alleviated mitochondrial superoxide production by reactive alkenals, but the mechanism is yet debatable (Brand et al., 2004).

MitoPQ is believed to disrupt the flavin site of Complex I mitochondria respiratory chain complex by redox cycling to produce ROS, called mitochondrial ROS (mtROS) (Robb et al., 2015). The interaction between ROS and this redox cycle mitochondrial membrane potential is known as “Reactive Oxygen Species (ROS)-induced ROS-release” (RIRR) (Zorov et al., 2006). Further disruption of the inner mitochondrial membrane aggravates the dysfunction of the inner membrane’s anion channel hence promoting mitochondrial swelling and thermoregulatory defective (Garlid and Beavis, 1986). Certain neurological diseases such as Alzheimer’s, Parkinson’s, Huntington’s disease and amyotrophic lateral sclerosis are also strongly related to mitochondria ROS production (Indo et al., 2015).

4.2 Aims and Objectives

Data in the previous chapter demonstrated the presence of Signal 1 and Signal 2 components of the NLRP3 inflammasome in THP-1 cells. The current chapter aimed to further investigate one of the published main mechanisms promoting inflammasome activation, that is, mitochondrial signals. Previous studies showed the effect of mitochondrial dysfunction towards Signal 2 activation and this occurred via ROS production. However, currently there is a lack of literature about mitochondrial targeted superoxide generators triggering inflammasome activation.

Both pro-IL-1β and pro-caspase-1 were used to investigate Signal 1 production and IL-1β and IL-18 for Signal 2 production. Additionally, caspase-5 was studied to delineate the possible involvement of the non-canonical pathway of NLRP3 inflammasome activation.

4.3 Experimental design

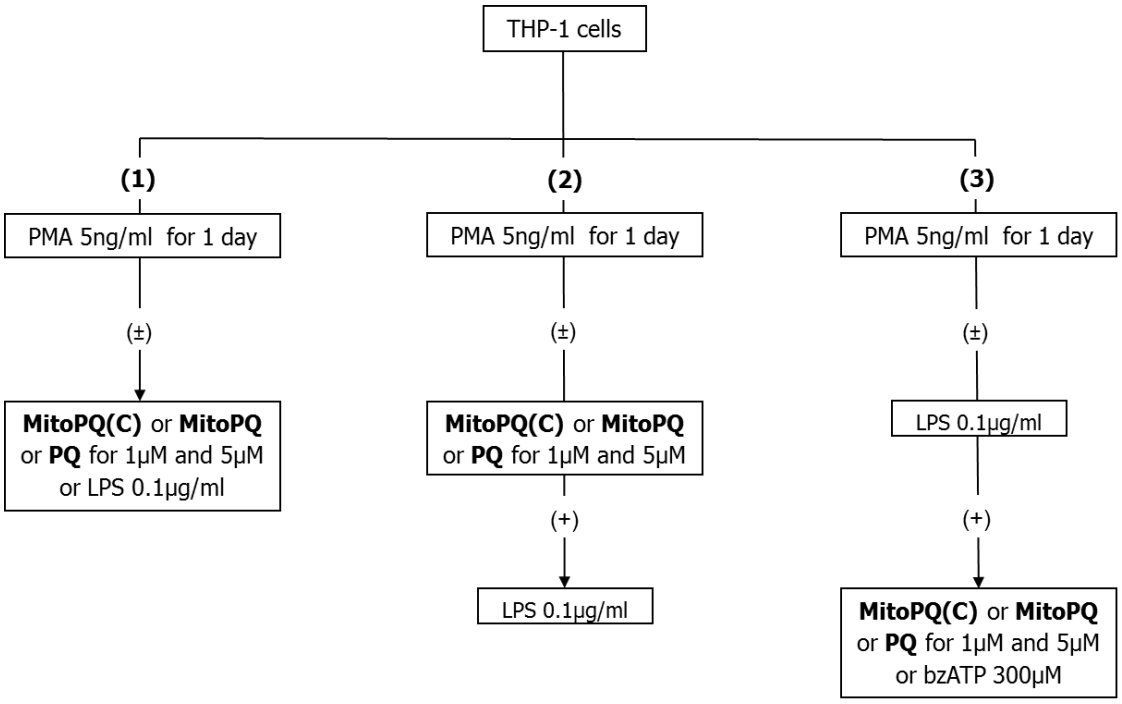

Figure 4-2 Flow chart investigating the effect of intracellular superoxide generation on THP-1 cell NLRP3 inflammasome.

Flow diagram shows step-by-step treatments of THP-1 cellss; either replacing LPS, prior to LPS or after the LPS administration.

4.4 Results

4.4.1 Superoxide generation by mitoPQ and PQ

Initially, the generation of superoxide by PQ and mitoPQ was investigated in THP-1 cells. Cells were stained with 200nM Rhodamine-based dye (mitoSOX) for 30 minutes at the end of each treatment (Jhang et al., 2015). Both mitoPQ and PQ obviously promoted mitoSOX fluorescence suggesting increased mitochondrial ROS production (Zhu et al., 2015).

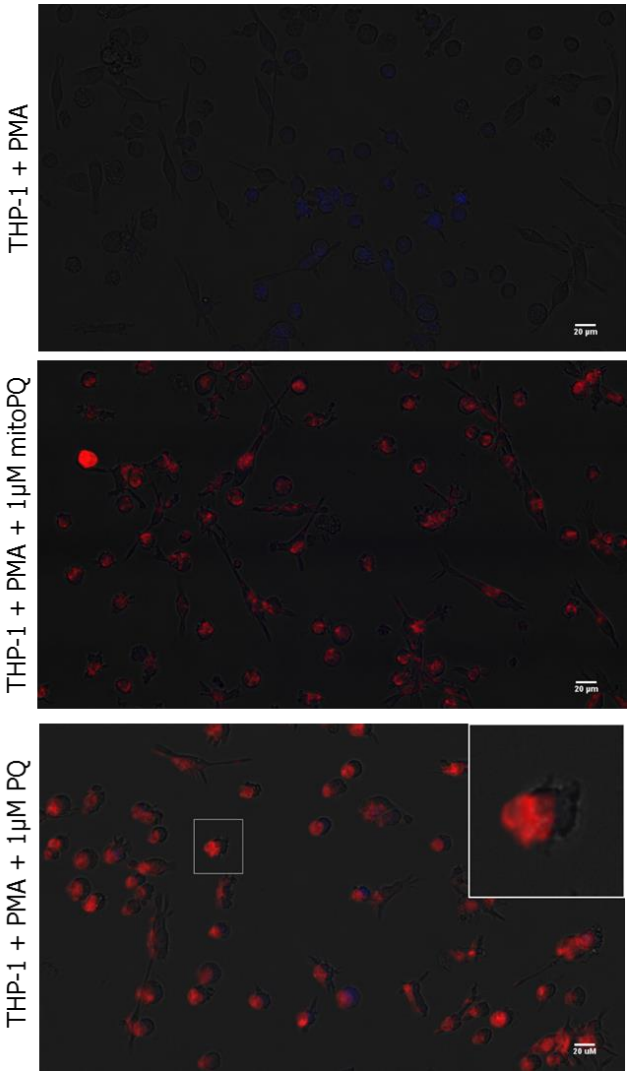

Figure 4-3 Fluorescence photomicrographs of mitoSOX-treated differentiated THP-1 cells.

MitoSOX-positive cells (red stained) were observed following treatment with intracellular superoxide generators (mitoPQ and PQ). Images shown at 20X magnification and scale bars, 20µm. Inset shown at 4X magnification.

4.4.2 Does intracellular superoxide cause Signal 1 activation in differentiated THP-1 cells?

The aim was to investigate whether intracellular superoxide could replace LPS which is known to be a potent Signal 1 agonist. Three compounds were used: PQ, mitoPQ and mitoPQ control (mitoPQ (C)). The latter two are targeted to the mitochondria but the control compound does not generate superoxide anion (**Figure 4-1c**). PQ was also used as a non-organelle specific superoxide generator.

LPS treatment was substituted by low concentrations of intracellular superoxide arising from mitoPQ and PQ (1µM for 24 hours). This was similar to the duration of treatment with LPS (**Figure 4-4** and **Figure 4-5**). bzATP was not added as the focus was on signal 1 alone. There were no differences in expression of NLRP3 protein when PQ and mitoPQ were used compared to the positive control (PMA+LPS).

Even at a higher concentration of 5µM the expression of NLRP3 protein as well as pro-IL-1β was not exaggerated by intracellular superoxide generators as compared to LPS treatment (**Figure 4-6** and **Figure 4-7**).

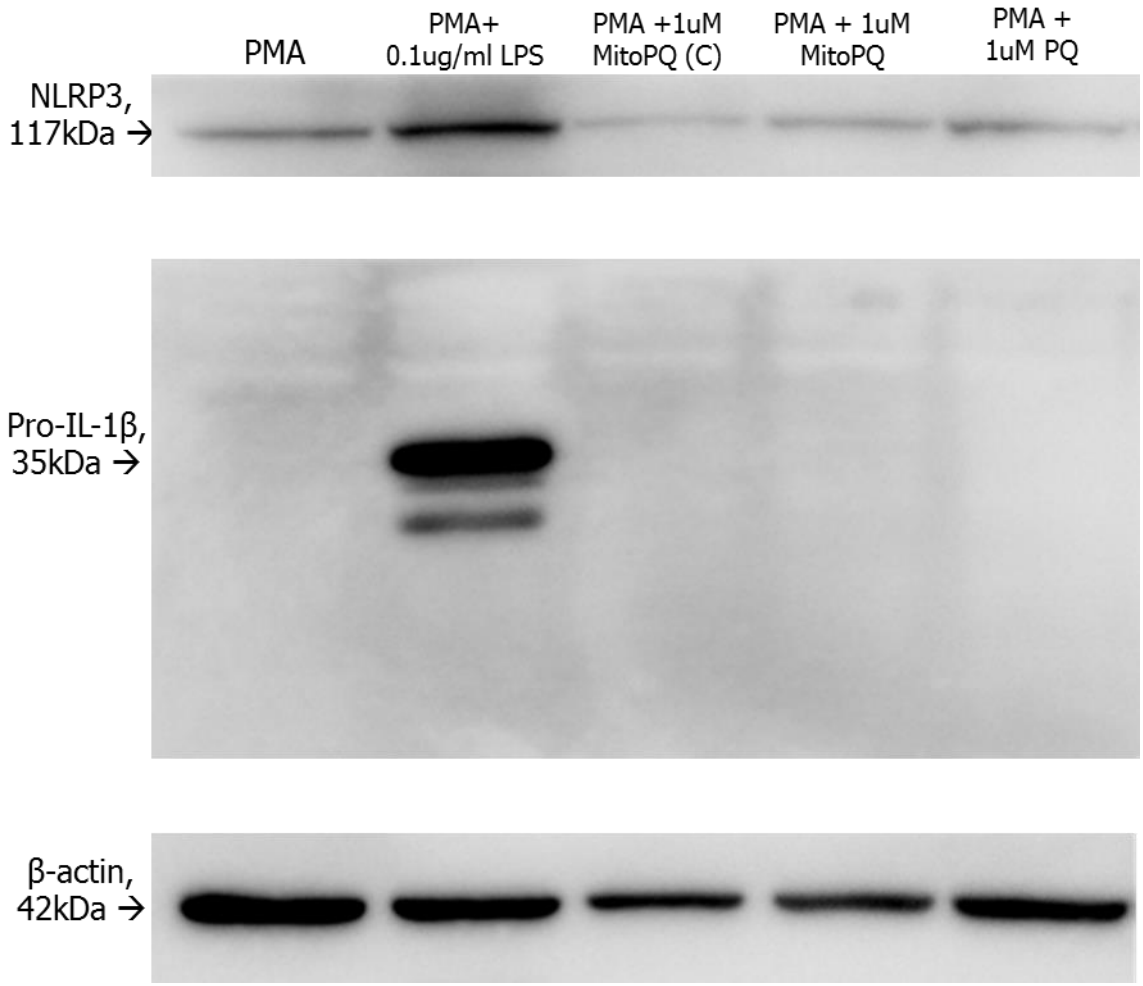

Figure 4-4 Effect of superoxide generators on Signal 1 expression in differentiated THP-1 cells.

Western blot showing the expression of NLRP3 and pro-IL-1β (Signal 1) in 50µg protein extracts of differentiated THP-1 cells. Differentiated THP-1 cells were treated with either mitoPQ (C), mitoPQ or PQ (1µM) for 24 hours or with LPS.

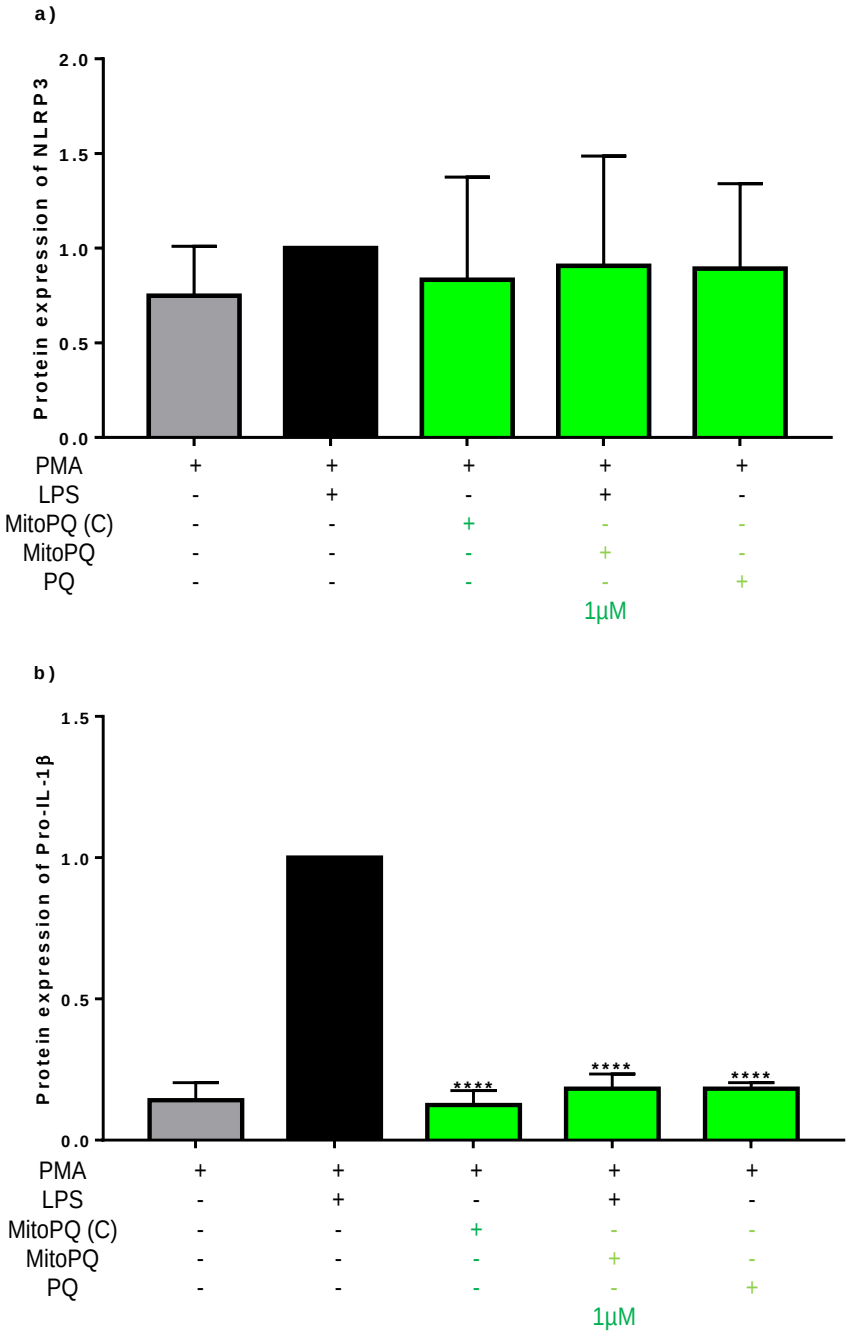

Figure 4-5 Quantitation of Signal 1 expression (NLRP3 and pro-IL-1β) in differentiated THP-1 cells.

Semi-quantitative Western blot showed the expression of Signal 1 after treatment with intracellular superoxide, replacing the LPS. There was no change in NLRP3 expression as compared to positive control. However, significant reduction of pro-IL-1β expressions were noted. Data presented as mean ± SEM, n=3, relative to β-actin (****$p<0.0001$ compared to PMA+LPS treatment) by ANOVA.

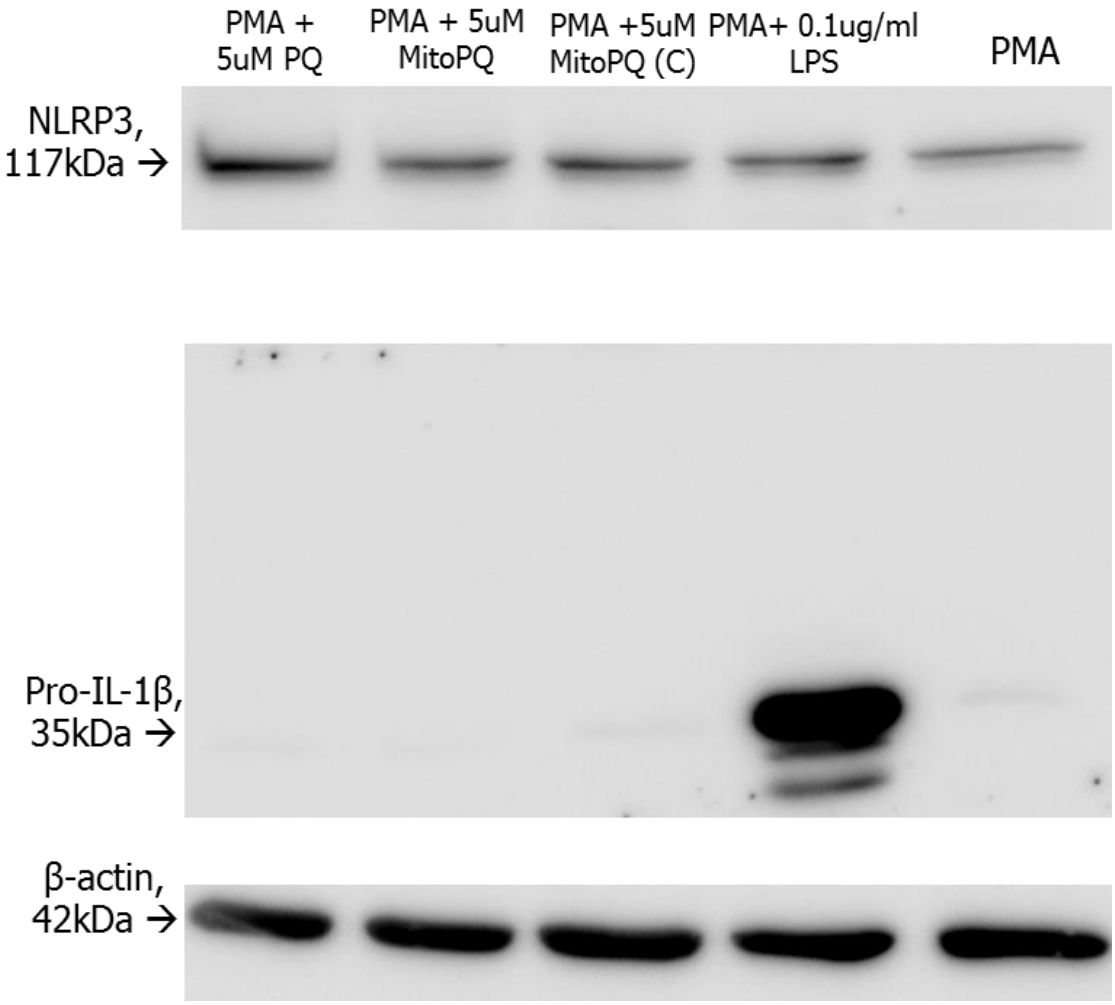

Figure 4-6 Effect of higher superoxide generators on Signal 1 expression in differentiated THP-1 cells.

Western blot showing the expression of NLRP3 and pro-IL-1β (Signal 1) in 50µg protein extracts of differentiated THP-1 cells. Differentiated THP-1 cells were treated with either mitoPQ (C), mitoPQ or PQ (5µM) for 24 hours or with LPS.

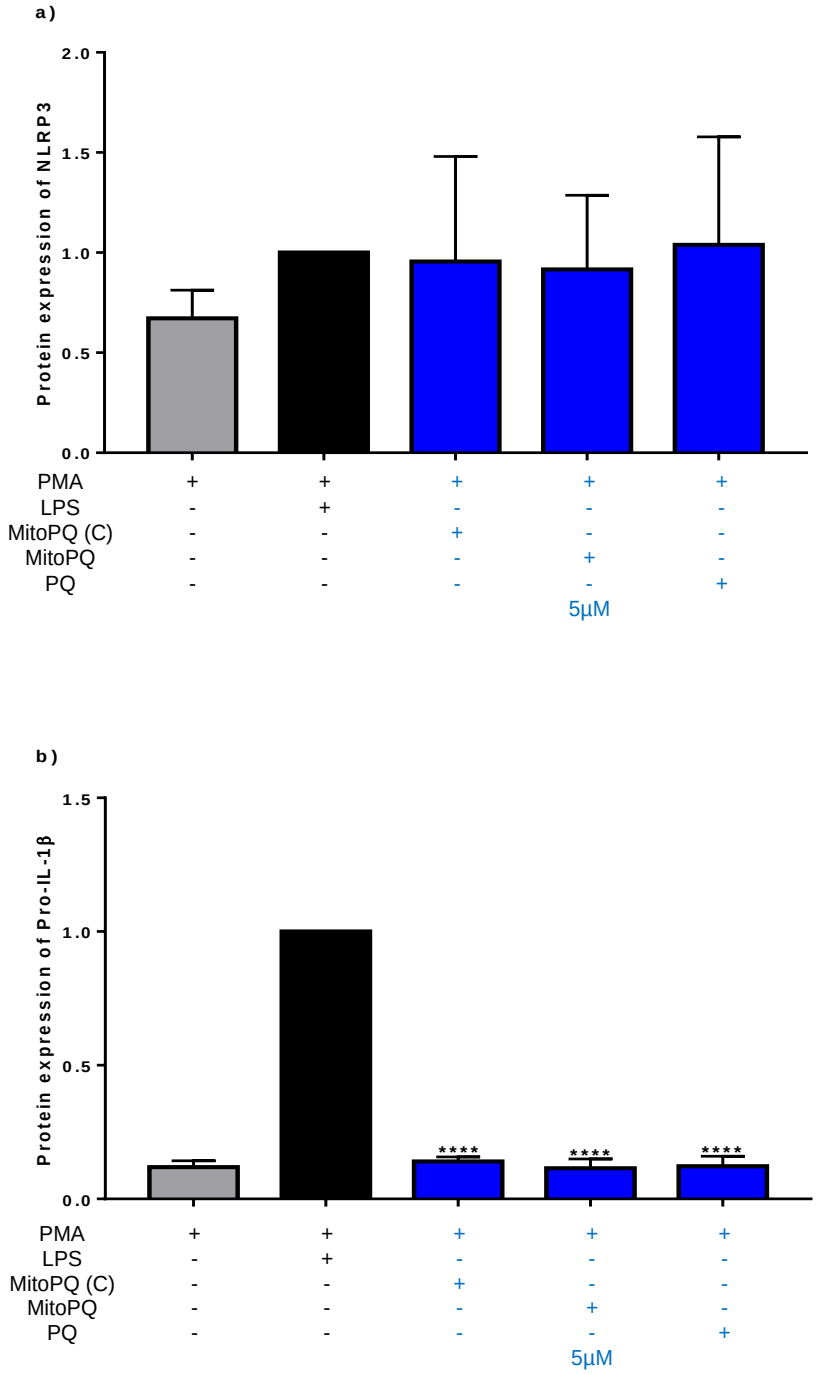

Figure 4-7 Quantitation of Signal 1 expression (NLRP3 and pro-IL-1β) in differentiated THP-1 cells.

Semi-quantitation Western blot showed the lack of expression of Signal 1 after treatment with higher concentration of intracellular superoxide, replacing the LPS (**b**). Data presented as mean ± SEM, n=3, relative to β-actin (****p<0.0001 compared to PMA+LPS treatment) by ANOVA.

4.4.3 Effect of intracellular superoxide generation on LPS-induced Signal 1 NLRP3 inflammasome in differentiated THP-1 cells

For this experiment, the effects of intracellular superoxide generator prior to LPS administration were studied. The aim of this treatment was to investigate whether mitoPQ or PQ enhance the Signal 1 induced by LPS.

Neither low concentrations (1µM) nor high concentration (5µM) of intracellular superoxide generator affected Signal 1 induction by LPS compared to positive control (PMA+LPS) (**Figure 4.8** and **Figure 4-9**).

We further investigated the production of caspase-1 in view of its role in Signal 2 production (**Figure 4-10** and **Figure 4-11**). None of the treatments increased caspase-1 expression compared to the control (PMA+LPS).

In summary, the intracellular superoxide generators were not able to enhance LPS-induced NLRP3 inflammasome priming in differentiated THP-1 cells.

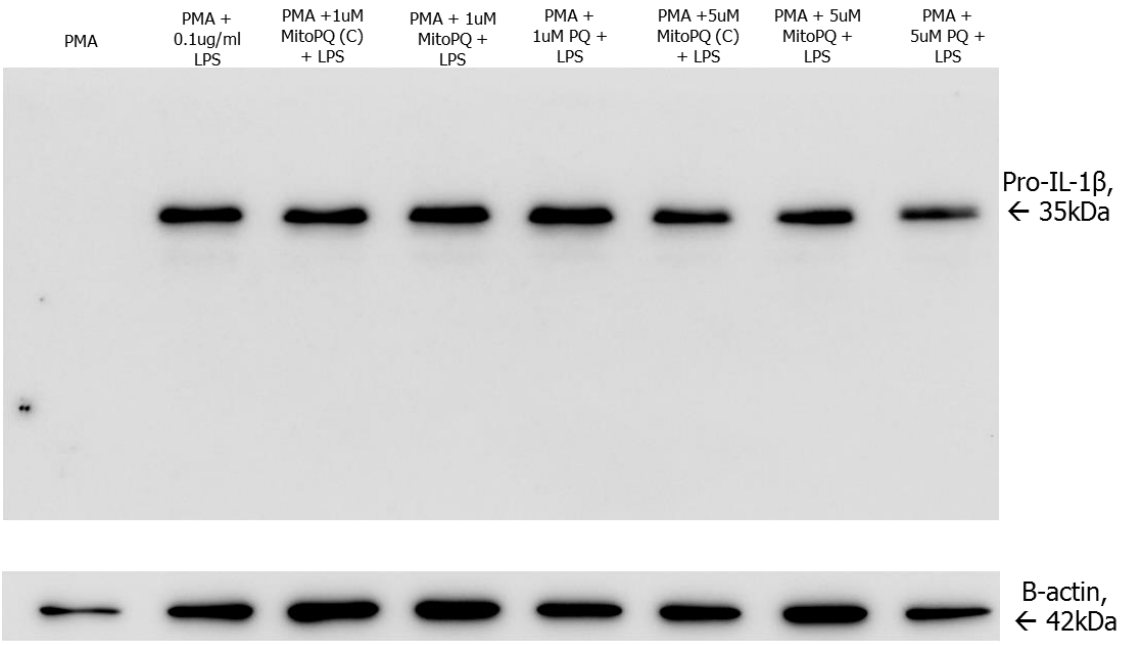

Figure 4-8 Effect of superoxide generators on Signal 1 expression in differentiated THP-1 cells.

Western blot showing the expression of pro-IL-1β (Signal 1) in 50µg protein extracts of differentiated THP-1 cells. Differentiated THP-1 cells were treated with either mitoPQ (C), mitoPQ or PQ (1µM) for 24 hours prior to LPS administration.

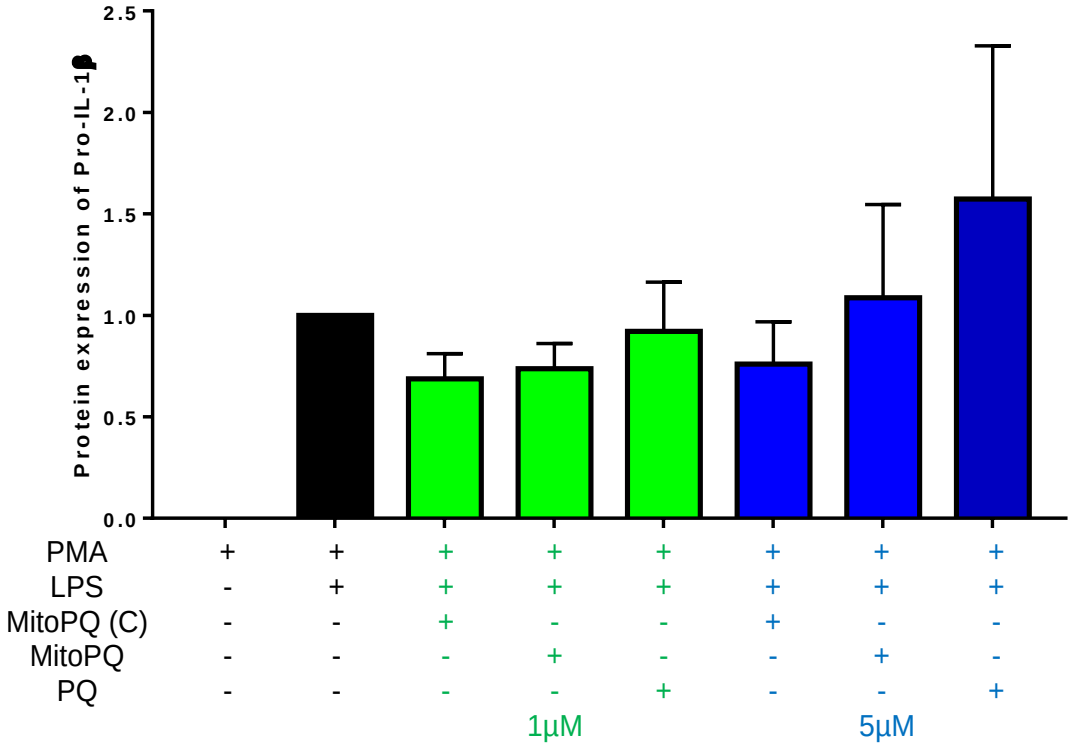

Figure 4-9 Quantitation of Signal 1 expression (pro-IL-1β) in differentiated THP-1 cells.

Semi-quantitative Western blot showed the expression of Signal 1 after treatment with intracellular superoxide, 1 μM and 5μM, respectively prior to LPS administration. There were no significant differences of Signal 1 expression compared to PMA+LPS by ANOVA. Data presented as mean ± SEM, n=3, relative to β-actin.

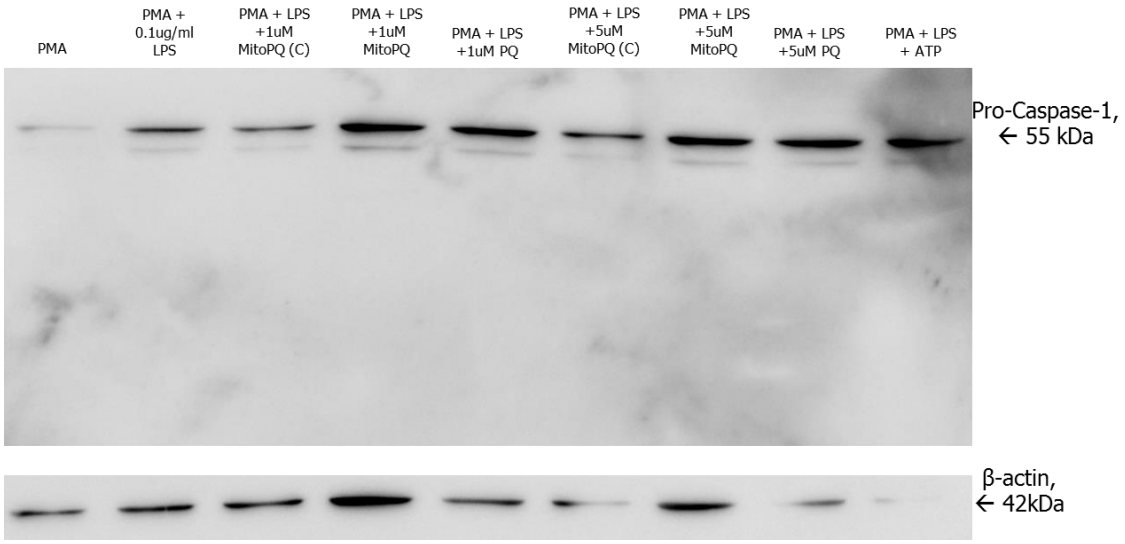

Figure 4-10 Effect of superoxide generators on pro-caspase-1 expression in differentiated THP-1 cells.

Western blot showed the expression of pro-caspase-1 (Signal 1) in 50µg differentiated THP-1 cells. Differentiated THP-1 cells were treated with either mitoPQ (C), mitoPQ or PQ, for 1µM and 5µM respectively for 1 hour, after the LPS administration for 24 hours.

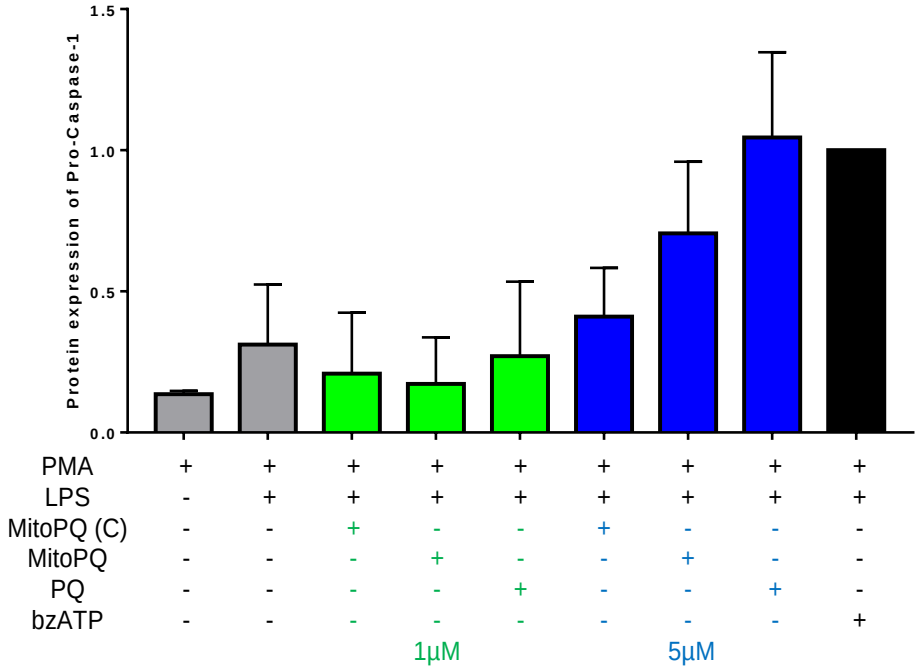

Figure 4-11 Quantitation of pro-caspase-1 in differentiated THP-1 cells, by 1µM and 5µM of PQ, mitoPQ and mitoPQ (C)

Quantitation of Western blot showed the expression of Signal 1 (pro-caspase-1) after treatment with intracellular superoxide, 1 µM and 5µM, respectively after the LPS administration. There was no significant changes of pro-caspase-1 expression in both dosage of PQ and mitoPQ treatment as compared to positive control (PMA+LPS) by ANOVA. Data presented as mean ± SEM, n=3, relative to β-actin.

4.4.4 Can superoxide act as Signal 2 for NLRP3 inflammasome activation in LPS-primed differentiated THP-1 cells?

The effect of intracellular superoxide generation as a replacement for bzATP stimulation of Signal 2 was investigated in differentiated and primed THP-1 cells. ELISA was performed in this study to investigate the release of active IL-1β and active IL-18 in the cell culture media.

As expected, bzATP caused a reduction in pro-IL-1β expression as determined by Western blotting (**Figure 4-12**) probably due to cleavage of the pro- form to active IL-1β. However, neither concentration of PQ or mitoPQ appeared to change the expression of pro-IL-1β as observed by Western analysis (**Figure 4-13**).

Further investigation of Signal 2 expression by ELISA revealed reduced production of active IL-1β (**Figure 4-14a**) and active IL-18 (**Figure 4-14b**) using either concentration of intracellular superoxide generators as compared to the potent Signal 2 inflammasome activator bzATP (positive control, PMA+LPS+bzATP). However, a statistically significant increase in IL-1β production was observed following mitoPQ (5μM) treatment, $p<0.05$ versus PMA+LPS control (**Figure 4-14a**).

In conclusion, there was some evidence that mitochondrial superoxide could enhance Signal 2 expression of LPS-treated differentiated THP-1 cells.

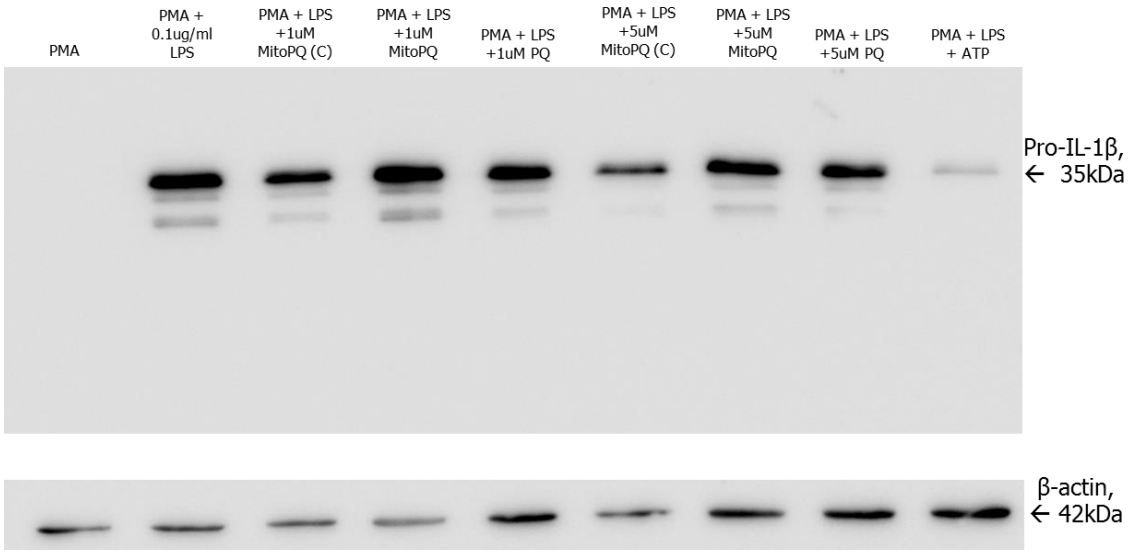

Figure 4-12 Effect of superoxide generators on Signal 1 expression in differentiated THP-1 cells.

Western blot showing the expression of NLRP3 and pro-IL-1β (Signal 1) in 50µg protein extracts of differentiated THP-1 cells. Differentiated THP-1 cells were treated with either mitoPQ (C), mitoPQ or PQ (1µM and 5µM) respectively for 1 hour, after LPS administration for 24 hours.

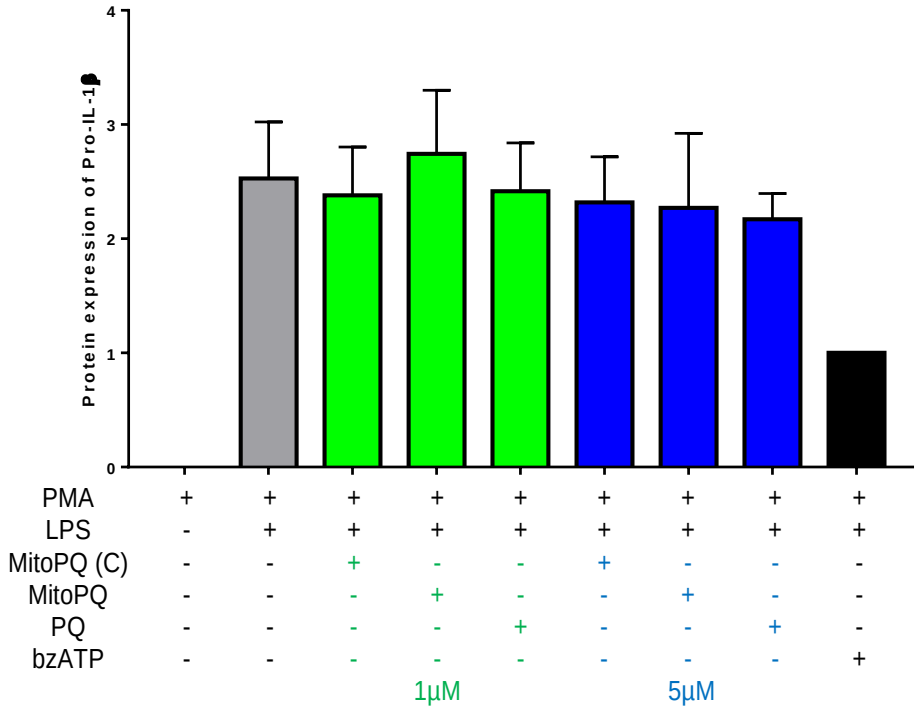

Figure 4-13 Quantitation of Signal 1 expression (pro-IL-1β) in differentiated THP-1 cells.

Quantitation of Western blot showed the expression of Signal 1 after treatment with intracellular superoxide generator, (1 μM and 5μM), after LPS administration. There was no significant increase of pro-IL-1β expression for either concentration of PQ and mitoPQ treatment as compared to positive control (PMA+LPS) by ANOVA. Data presented as mean ± SEM, n=3, relative to β-actin.

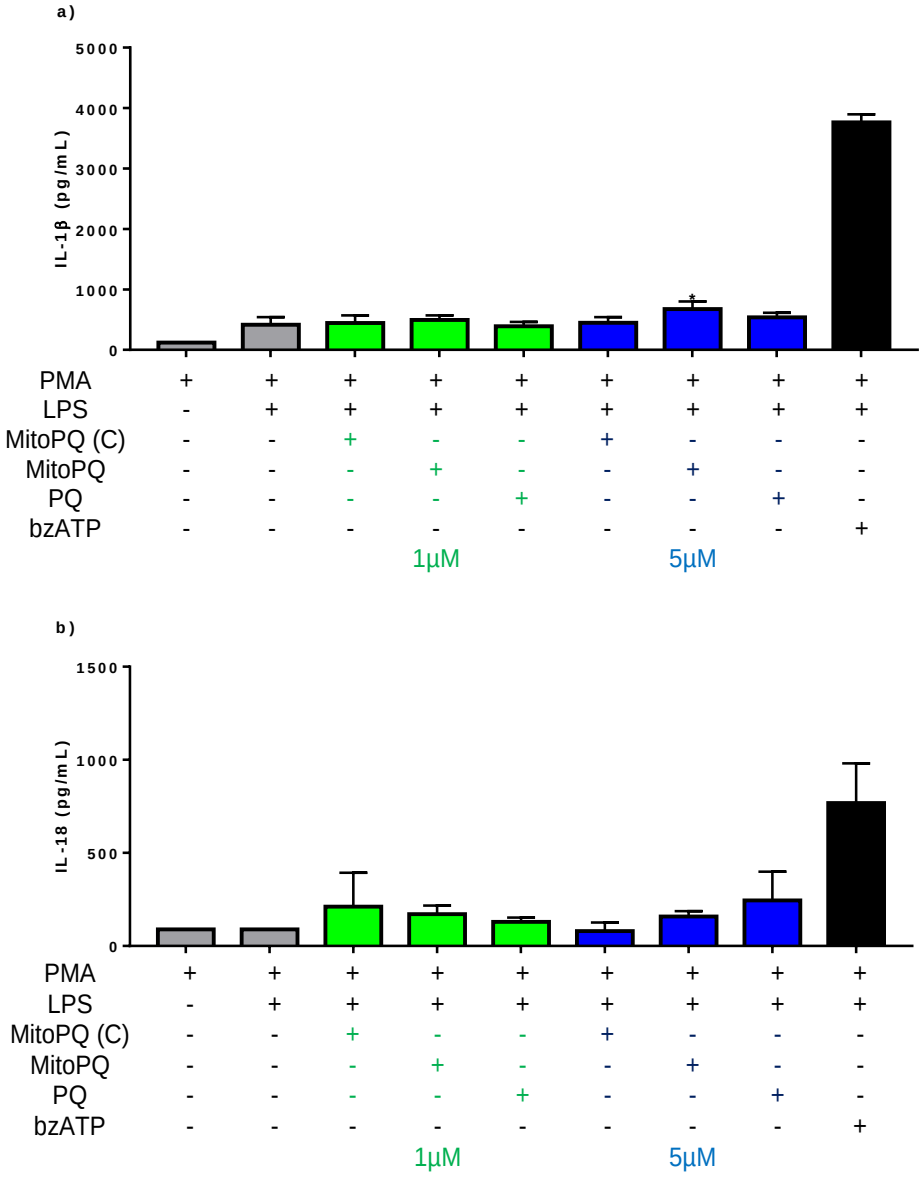

Figure 4-14 Signal 2 expression in cell culture media of differentiated THP-1 cells.

Results showed significant increased Signal 2 expression; active IL-1β (**a**) by bzATP and 5µM mitoPQ in differentiated THP-1 cells as compared to cells treated with PMA+LPS alone. However, there was no significant differences in IL-18 expression (**b**). *p<0.05 compared to PMA+LPS by ANOVA. Data presented as mean ± SEM, n=3.

4.5 Discussion

4.5.1 Intracellular superoxide generation and NLRP3 inflammasome Signal 1

As mentioned previously, Signal 1 of the inflammasome, or priming, involves NF-κB activation, either by transcriptional, translational or post translational level processes hence producing NLRP3 protein, pro-IL-1β and pro-IL-18 (Hoseini et al., 2017). Subsequent oligomerization of the inflammasome complex creates the receptor (NLRP3), adaptor (ASC) and effector (active caspase-1) resulting in the cleavage of pro-proteins forming active IL-1β and IL-18 (Signal 2).

Interestingly, a previous study demonstrated the relationship of ROS with Signal 1 activation, especially via the activation of NF-κB (Chandel et al., 2000). However, there are no studies on the effects of mitoPQ and PQ towards activation of Signal 1 inflammasome. These intracellular superoxide generators were studied to demonstrate the direct effect towards the mitochondria.

Two different concentrations of PQ and mitoPQ were used in this experiment based on a previous study (Robb et al., 2015). The first experiment was to demonstrate the effectiveness of intracellular superoxide generators in replacing LPS treatment.

Neither concentration of superoxide generator resulted in Signal 1 activation. A previous study noted triggering of Signal 1 by death-associated protein kinase (DAPK) and NF-κB by paraquat (Liu et al., 2017). However, this study was performed *in vivo* with high concentration of PQ. Study of the NADPH oxidase pathway also might be helpful in investigating the intracellular superoxide generator on Signal 1 activation (Albornoz et al., 2016).

Another study has suggested that the ROS effect of PQ and mitoPQ was limited with respect to replacing LPS as second messenger for Signal 1 activation. This effect corresponded to our study (Mathy-Hartert et al., 2003). Further study of other family members of transcription factors which related to NF-κB-ROS interaction such as activator protein 1 (AP-1), specificity protein 1 (Sp1) and

peroxisome proliferator-activated receptors (PPARs) might be worthy of investigating in order to study whether ROS activate the inflammasome (Lavrovsky et al., 2000).

A previous study in LPS-treated mice demonstrated that ROS inhibitor; N-acetyl-L-cysteine (NAC) down-regulates the Signal 1 of NLRP3 inflammasome (Bauernfeind et al., 2011). This study certainly showed the effect of superoxide production in stimulating Signal 1 NLRP3 inflammasome. However, a higher concentration of LPS (0.2µg/ml) was used as compared to our study. Additionally, the mechanism involving pro-inflammatory TNF-α activation in superoxide model might be feasible to relate with the Signal 1 production (Alvarez-Erviti et al., 2011).

Furthermore, another study showed that Signal 1 is regulated by superoxide production via mitochondrial ROS (mtROS) activation by regulating its transcriptional induction. However, primary bone marrow macrophages were used in this study instead of *in vitro* study (Juliana et al., 2012). Additionally, this study also demonstrated the combination of ATP and LPS to induce mtROS, which might be beneficial for our further research on superoxide induced Signal 1 inflammasome.

Surprisingly, there was a study in mice showing that low concentrations of mitoPQ (<1nM) were cardioprotective against myocardial infarction hence mimicking anti-inflammatory effects (Mulvey et al., 2017). This study demonstrated that a low concentration of mitoPQ production was protective against acute myocardial infarction. This study emphasized the protective response rather than a preventive response.

Additionally, a previous study using PQ showed inhibition of LPS-induced lymphocyte proliferation, interferon (IFN)-γ production, and monocyte phagocytosis and less Signal 1 NLRP3 inflammasome was observed (Jang et al., 2015). However, higher concentrations of PQ were used in this study (75µM and 150µM).

Even though there were no obvious differences in pro-caspase-1 activation by PQ or mitoPQ, this could be explained by data from a previous study on the activation of pro-caspase-1 being related with AIM2 inflammasome (Hornung et al., 2009). Further investigation of natural killer cell–dependent production of IFN-γ might be needed to investigate the production of pro-caspase-1 in NLRP3 inflammasome pathway during superoxide generation by mtROS (Rathinam et al., 2010). Measurement of active caspase-1 might be helpful.

4.5.2 Intracellular superoxide generation and Signal 2 NLRP3 inflammasome

Paraquat and mitoparaquat are known to disturb mitochondrial respiratory chain Complex 1 as redox cyclers and produce reactive oxygen species and lipid peroxidation (Finkel, 2005). These intracellular superoxide generators or so-called DAMPs triggered mitochondrial H_2O_2 and promoted inflammation by NLRP3 inflammasome production in mice (Chen et al., 2015). One study has mentioned about the relationship of PQ and mitoPQ targeting the NLRP3 inflammasome but the exact pathways involved are not elucidated (Liu et al., 2015)

A previous study mentioned about the production of IL-1β by human mononuclear cells by PQ poisoning after LPS administration. However, a higher concentration of PQ (100μM) was used which might disturb the whole cell rather than specifically mitochondria (Erroi et al., 1992).

Additionally, study of PQ poisoning and IL-18 production has shown previously that superoxide anions are generated triggering P38 MAPK phosphorylation and ERK 1/2 pathways activating caspase-1 hence production of active IL-18 (Sekiyama et al., 2005). Therefore, further experiments might be needed to increase “n” number beyond that possible in this thesis.

Interestingly, mitoPQ slightly stimulated Signal 2 NLRP3 inflammasome as predicted. A study showed that mitoPQ is several hundred-fold more potent at producing hydrogen peroxide as compared to PQ and produces superoxide by

redox cycling at the flavin site of Complex I in mitochondria (Cochemé and Murphy, 2008). However, the increased IL-1β production might be due to direct targeting of mitochondria by the compound rather than by the redox cycler effect. MitoPQ(C) did not stimulate Signal 2 activation, suggesting that mere accumulation of compounds within mitochondria due to mitochondrial membrane potentials is not sufficient to activate NLRP3 inflammasome. However, mitoPQ did not increase IL-1β synthesis compared to mitoPQ(C) and so further experimentation is required to fully explore the mechanism. Further investigation by uncoupler FCCP (carbonyl cyanide p-(tri-fluromethoxy) phenyl-hydrazone) might be beneficial to determine whether the energized mitochondria is directly triggered by the mitoPQ via redox cycling at Complex 1 (Robb et al., 2015).

Furthermore, there was a trend for increased IL-18 due to PQ and mitoPQ in LPS-treated THP-1 differentiated cells. A previous study demonstrated that higher expression of IL-18R (receptor) by ROS was mediated by transcription factors such as NF-κB, MAPKs and STAT1 (signal transducer and activator of transcription 1) (Zaki et al., 2010). It might be that both IL-18 and its receptor are upregulated by ROS, but this remains to be proven (Felderhoff-Mueser et al., 2005).

4.5.3 Intracellular superoxide generation in non-canonical NLRP3 inflammasome pathway activation

Based on the non-stimulus of active caspase-1 expression, we then proceeded with investigations of different pathways that might be involved in IL-1β activation, for example the non-canonical pathway of NLRP3 inflammasome. This pathway involves caspase-11 pathway activation in mice and caspase 4 or 5 pathways in humans (Kayagaki et al., 2013) resulting in pyroptotic cell death.

Additionally, this unconventional pathway mainly appears in monocytic cells which need LPS stimulation. Even though caspase 4 stimulation may remain uncleaved, caspase-5 cleavage is believed to undergo rapid processing upon LPS treatment which was investigated in this chapter (Vigano et al., 2015).

We stimulated THP-1 cells with intracellular superoxide generator before and after LPS stimulation, respectively. However, none of them showed either pro-caspase-5 or active caspase-5 expression (data not shown). These findings might tally with a previous study mentioning the need of caspase-1 in processing IL-1β. As a result, pro-IL-1β is cleaved by caspase-1 activation rather than the caspase-5 non-canonical NLRP3 dependent pathway (Kang et al., 2000).

A positive control using etoposide treated HeLa cells was used to validate the anti-caspase-5 antibody. However, no band was observed on the Western blot. This raises doubt as to the specificity of the antibody and so further study would be required.

4.6 Conclusions

In conclusion, intracellular superoxide generators (PQ and mitoPQ) were unable to enhance Signal 1 (NLRP3, pro-IL-1β and pro-caspase-1) expression in differentiated THP-1 cells.

Interestingly, evidence was presented that mitoPQ was able to increase Signal 2 activation in differentiated, primed THP-1 cells.

Non-canonical pathway (pro-caspase-5) was not detected in THP-1 cells in this study.

Further work on other compounds which antagonise the mitochondrial ROS production or preserve mitochondrial integrity, such as hydrogen sulphide donors (GYY4137 and AP39) might be helpful in studying the role of redox balance in mitochondria towards NLRP3 inflammasome activation, and provide possible avenues for protection against pro-inflammatory effects of mtROS. This possibility is addressed in Chapter 5.

Chapter 5 Investigation of the effect of H_2S generation on NLRP3 inflammasome activation

5.1 Background

5.1.1 Hydrogen sulfide and the NLRP3 inflammasome

H_2S is one of the gasotransmitter molecules which is produced by three main enzymes: CBS and CSE in cytosol and 3MST in both cytosol and mitochondria (Kimura, 2011). Generally, H_2S has beneficial properties on the cardiovascular system such as vasodilator effects, anti-platelet activity and smooth muscle relaxation. Of the four classifications of H_2S donor, a hydrolysis-triggered H_2S donor is the most frequently used; one of the best studied is GYY4137 (Li et al., 2008).

The effect of H_2S and H_2S donors on inflammation is still debatable, particularly effects on the NLRP3 inflammasome (Whiteman and Winyard, 2011). However, most of the studies showed anti-inflammatory effects with slow-releasing H_2S donor (Li et al., 2013).

Interestingly, few studies have previously mentioned about the interactions of LPS and H_2S *in vivo* or *in vitro*. In 2005, LPS was shown to upregulate CSE levels hence increasing H_2S formation in tissues and plasma in mice, hence acting in an anti-inflammatory manner. Conversely, NaHS administration caused neutrophil infiltration and a pro-inflammatory response resulting in lung and liver damage, shown by increased myeloperoxidase (MPO) levels. It was demonstrated that NaHS activates the $MAPK^{p38}$/MK-2/Hsp27 pathway hence supporting TNF-α in promoting cytokine production (Li et al., 2005).

Slow and sustained-release H_2S donor, GYY4137 was compared with NaHS in LPS-induced macrophages. GYY4137 significantly inhibited pro-inflammatory responses such as PGE_2, TNF-α, CRP, L-selectin, IL-6 and IL-1β production in a concentration-dependent manner, consistently by NF-κB inhibition. Furthermore, the highest concentration of GYY4137, 1000μM, did not affect cell viability. Other than anti-inflammatory properties, this study also demonstrated hypotension effects as expected. It can be concluded that, slow-releasing compounds

releasing H_2S over an extended time were more effective as compared to fast-releasing compounds as an anti-inflammatory strategy. This might be related with activating transcription factor, ATF-2 and heat shock protein, HSP-27 (Whiteman et al., 2010).

Inflammation and oxidative stress have been indirectly implicated in high glucose-induced cardiomyopathy or diabetic cardiomyopathy (DCM) by disturbance of diastolic ability and cardiac flexibility. Subsequent exogenous H_2S treatment (NaHS) deactivated both Signal 1 and Signal 2 of the NLRP3 inflammasome pathway reducing IL-1β and IL-18 synthesis in cardiomyocytes (H9c2 cells) via TLR4/NF-κB inhibition. NaHS demonstrated reduction of ROS, decreased apoptosis rate and restored mitochondrial membrane potential (Huang et al., 2016).

Further a recent study mimicking periodontal disease patients in 2017 revealed pro-inflammatory effects of NaHS towards THP-1 cells and peripheral blood mononuclear cells (PBMCs). Nevertheless, this study only focused on signal 2 end products of the NLRP3 inflammasome, that is, IL-1β and IL-18. There were no findings reported with respect to Signal 1 activation. Surprisingly this study revealed that NaHS promotes Signal 2 activation in THP-1 cells without addition of a priming agent such as LPS , which might be possibly by the MAPK pathway (Basic et al., 2017). However, no *in vivo* effects were demonstrated in this study.

Recently *in vitro* studies involving the slow-releasing H_2S donor, GYY4137, showed potent pro-inflammatory effects in THP-1 and bone marrow-derived macrophage (BMDM) cells as compared to a fast-releasing H_2S donor, sodium thiosulfate (STS). This study demonstrated that inhibition of mitochondrial ROS production by H_2S donors prevented NLRP3 inflammasome activation, which is IL-1β and caspase-1 synthesis. Furthermore, the H_2S donor targeted a disulfide bond of the inflammasome directly by persulfhydration. Likewise, no Signal 1 or priming of NLRP3 inflammasome mechanism was shown in this study (Castelblanco et al., 2017).

Taken together, recent studies showed some effects of H_2S on NLRP3 inflammasome activation in human and rodent cell types. Nevertheless, the

effects are conflicting especially for GYY4137 where both pro- and anti-inflammatory effects are described. In particular, the effects of slow release H_2S donors on Signal 1 and the role for mitochondrial H_2S generation have not been well studied to date.

5.2 Aims and Objectives

Various studies demonstrated either pro- or anti-inflammatory effects of hydrogen sulfide donors. Furthermore, the different treatment concentrations are important to consider in determining the effects *in vivo* and *in vitro.* Interestingly, slow-releasing H_2S donors show potentially beneficial effects on NLRP3 inflammasome activation. However, there are limited studies on the effects of H_2S donors on NLRP3 inflammasome Signal 1, hence making the subject of interest to focus on especially investigating the molecular pathways.

Thus, the aim of this chapter is to investigate the effects of a slow-releasing H_2S donor (GYY4137) on inflammasome priming and activation in differentiated THP-1 cells. Additionally, mitochondria-targeted slow-releasing H_2S donor (AP39) was used to investigate whether the intracellular source of H_2S is important for modulation of NLRP3 inflammasome activation. NLRP3 protein and pro-IL-1β were used to investigate Signal 1 production whereas IL-1β and IL-18 were used to indicate for Signal 2 activation.

5.3 Experimental design

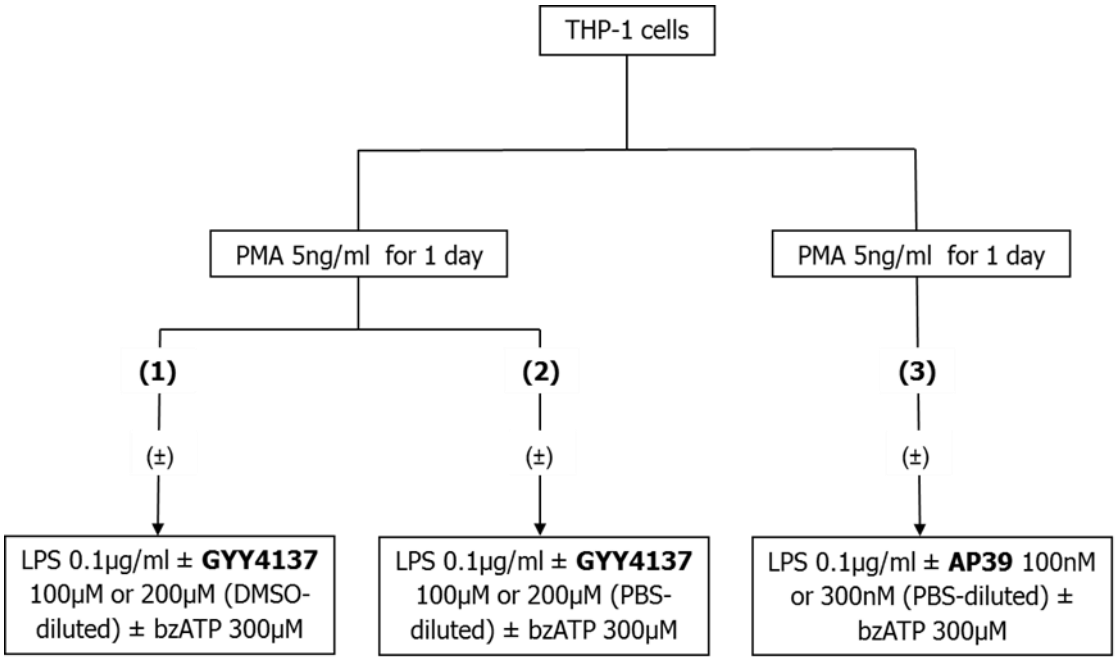

Figure 5-1 Flow chart investigating the effect of hydrogen sulfide donors on NLRP3 activation in THP-1 cells.

Flow diagram shows step-by-step treatments of THP-1 cells; either with GYY4137 (in DMSO- and PBS-diluted) or AP39.

5.4 Results

5.4.1 Effects of GYY4137 on Signal 1 and Signal 2 NLRP3 inflammasome activation in differentiated LPS-treated THP-1 cells

This study investigated the effect of GYY4137, a slow-releasing H_2S compound, towards differentiated THP-1 cells, following LPS treatment. The GYY4137 was dissolved in either DMSO or PBS. Two concentrations of GYY4137 were used in this experiment; 100µM and 200µM, as used in previous reported studies in inflammation research (Whiteman et al., 2010). As previously described, PMA (5ng/ml) was used to differentiate THP-1 to macrophages and LPS (0.1µg/ml) administered for Signal 1 activation. Lastly, bzATP was added to activate Signal 2 of the NLRP3 inflammasome to produce active IL-1β and IL-18.

Initially, GYY4137 dissolved in DMSO was added simultaneously with LPS, without bzATP (**Figure 5-2** and **Figure 5-3**). At either concentration, GYY4137 showed an approximately 2 fold reduction of pro-IL-1β as compared to the control ($p<0.001$) (**Figure 5-3b**). However, there were no obvious changes in NLRP3 expression by Western blotting.

Experiments were then conducted with addition of bzATP at the end of LPS treatment to observe the Signal 2effects of NLRP3 inflammasome (**Figure 5-4** and **Figure 5-5**), however, only Signal 1 effects were observed (NLRP3 and pro-IL-1β). Even though there was no significant changes in NLRP3 production, a significant increased of pro-IL-1β protein expression was noted on the treatment of low concentration of GYY4137 (100µM) in DMSO (**Figure 5-5b**).

It was then realised that the experiment with GYY4137 were not well controlled and that DMSO should be used alone. Results showed significantly reduced Signal 1 (pro-IL-1β) expression by DMSO alone, with or without bzATP (**Figure 5-6**). These data cast serious doubt on the results presented in **Figure 5-3** and **Figure 5-5.**

Further investigation with ELISA was carried out to investigate the Signal 2 production of the NLRP3 inflammasome. The lower concentration of DMSO

(0.4%) caused a statistically significant reduction in IL-18 level without bzATP administration. However, upon bzATP administration, an increase in IL-18 was observed (**Figure 5-7b**). A reduction in IL-18 was observed for the higher DMSO concentration (0.8%) following bzATP administration.

In the absence of bzATP activation IL-1β levels appeared reduced by DMSO significantly by lower concentration of DMSO (**Figure 5-7a**). Data for IL-1β with bzATP were above the working range of the assay and therefore have not been included in **Figure 5-7**.

Taken together, these data with DMSO demonstrate an anti-inflammasome effect in LPS-treated differentiated THP-1 cells making it difficult to interpret previous findings for GYY4137. Hence, PBS-diluted GYY4137 was used in subsequent experiments.

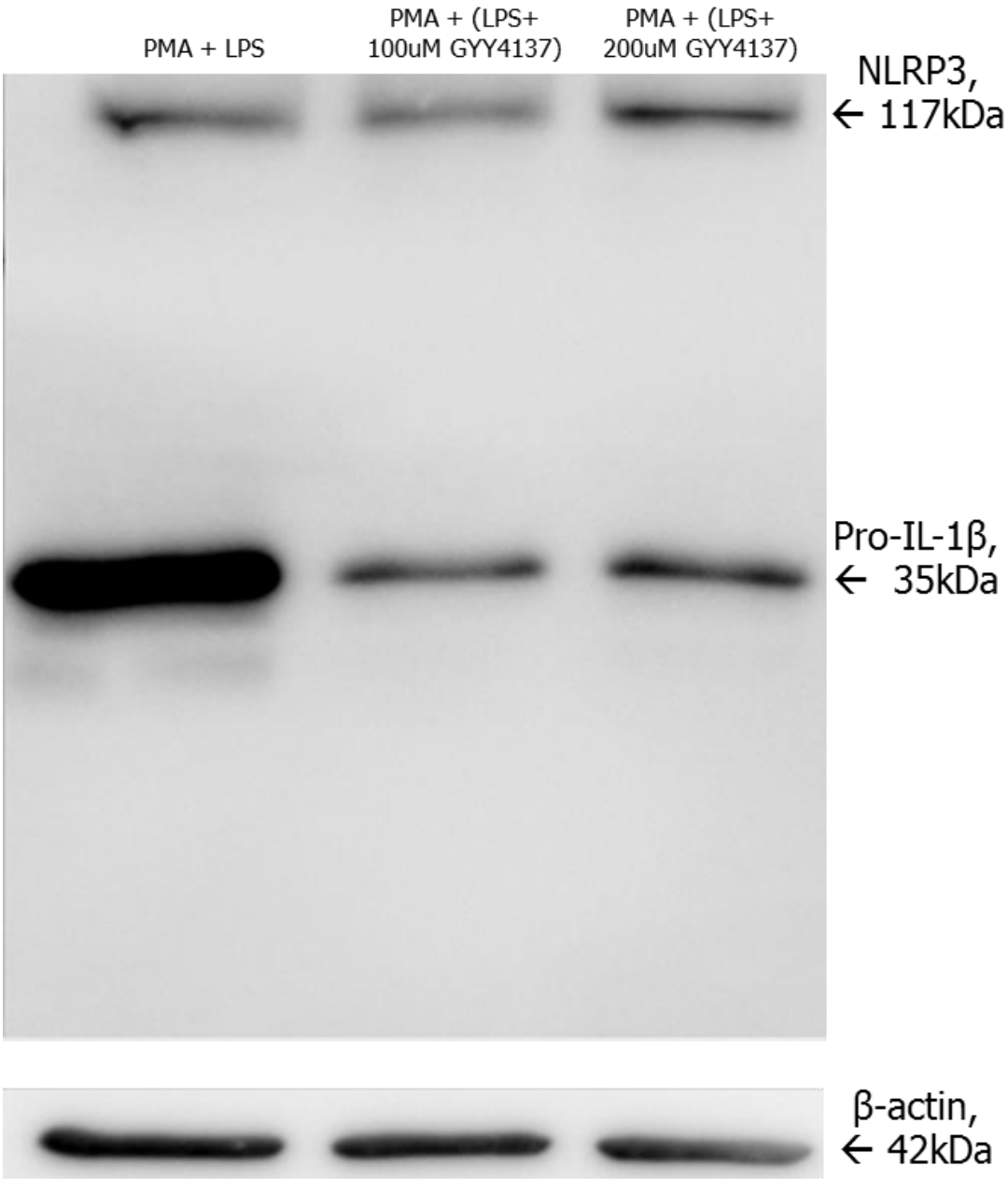

Figure 5-2 Effect of GYY4137 on NLRP3 inflammasome Signal 1 in differentiated THP-1 cells.

Western blot expression of NLRP3 and pro-IL-1β (Signal 1) in 50µg of differentiated THP-1 cell lysates. Differentiated THP-1 cells were treated with LPS with or without GYY4137 for 24 hours.

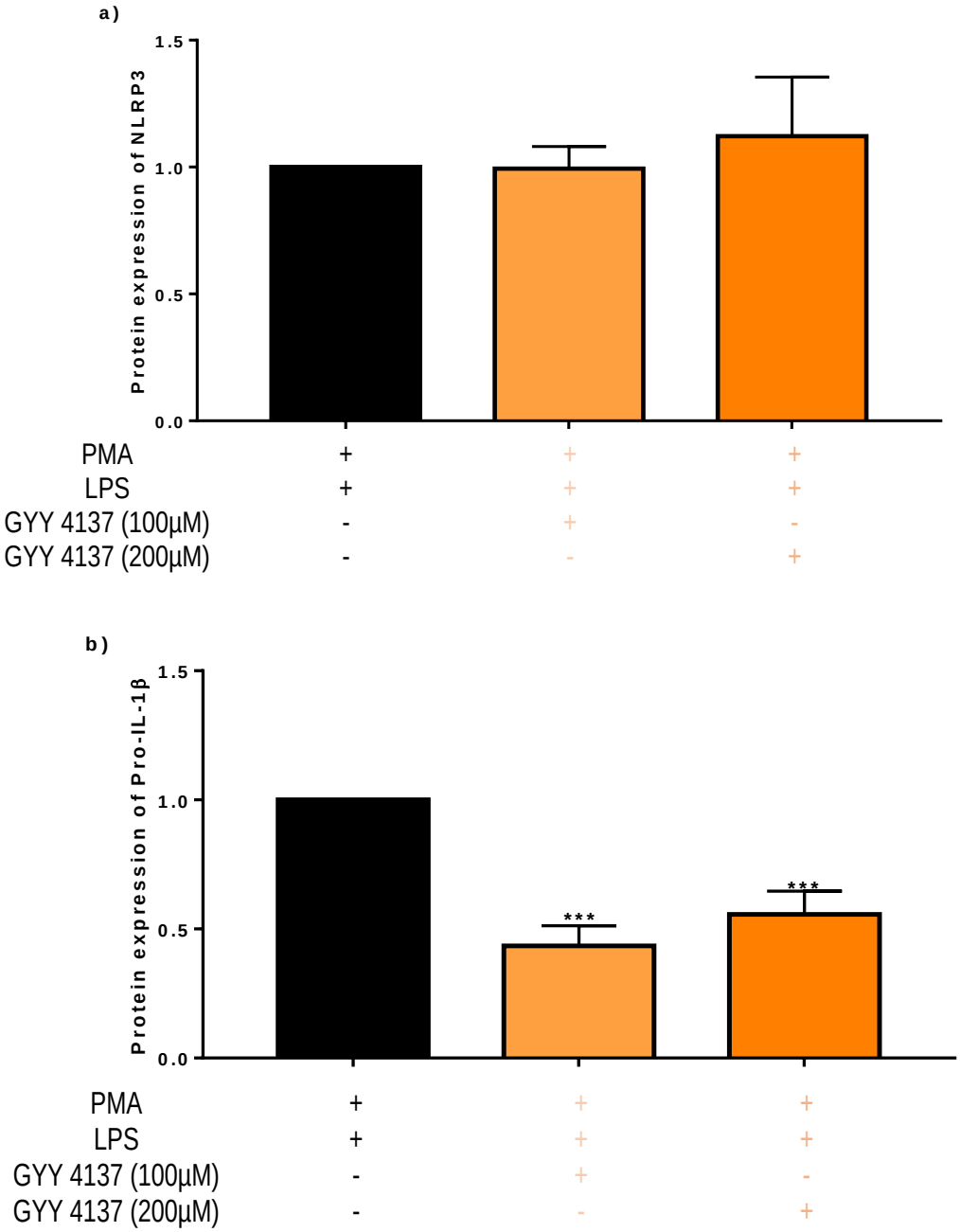

Figure 5-3 Effect of GYY4137 on NLRP3 inflammasome Signal 1 in THP-1 cells.

Western blot bands for NLRP3 (**a**) and pro-IL-1β (**b**) were analysed by densitometry relative to β-actin housekeeper. GYY4137 reduced pro-IL-1β (***p<0.001 compared to PMA+LPS treatment). Data presented as mean ± SEM, n=3. Statistical analysis (ANOVA) was used to compare treated cells with PMA+LPS control.

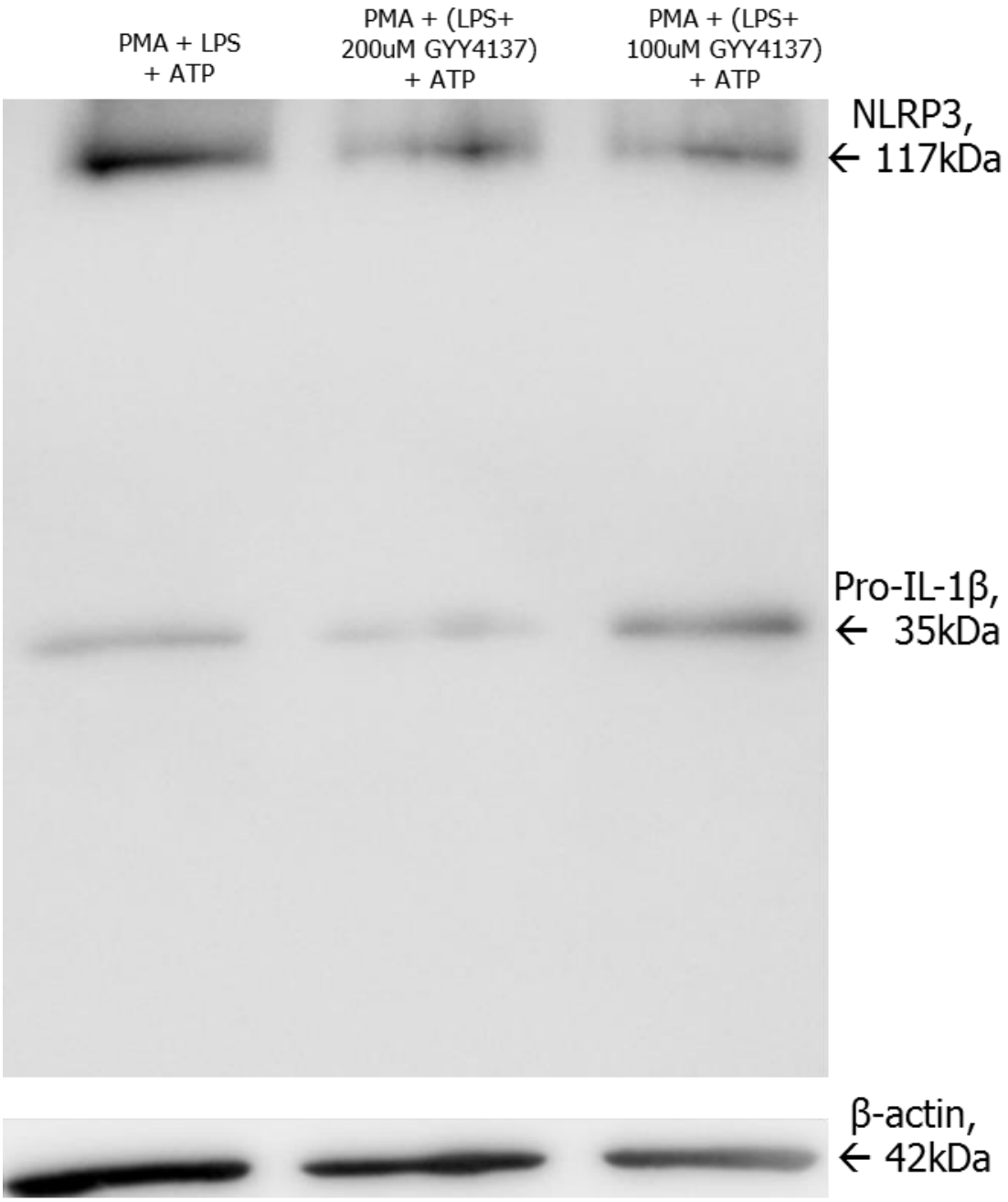

Figure 5-4 bzATP effect of GYY4137 on NLRP3 inflammasome Signal 1 in differentiated THP-1 cells.

Western blot expression of NLRP3 and pro-IL-1β (Signal 1) in 50µg of differentiated THP-1 cell lysates. Differentiated THP-1 cells were treated with LPS with or without GYY4137 for 24 hours.

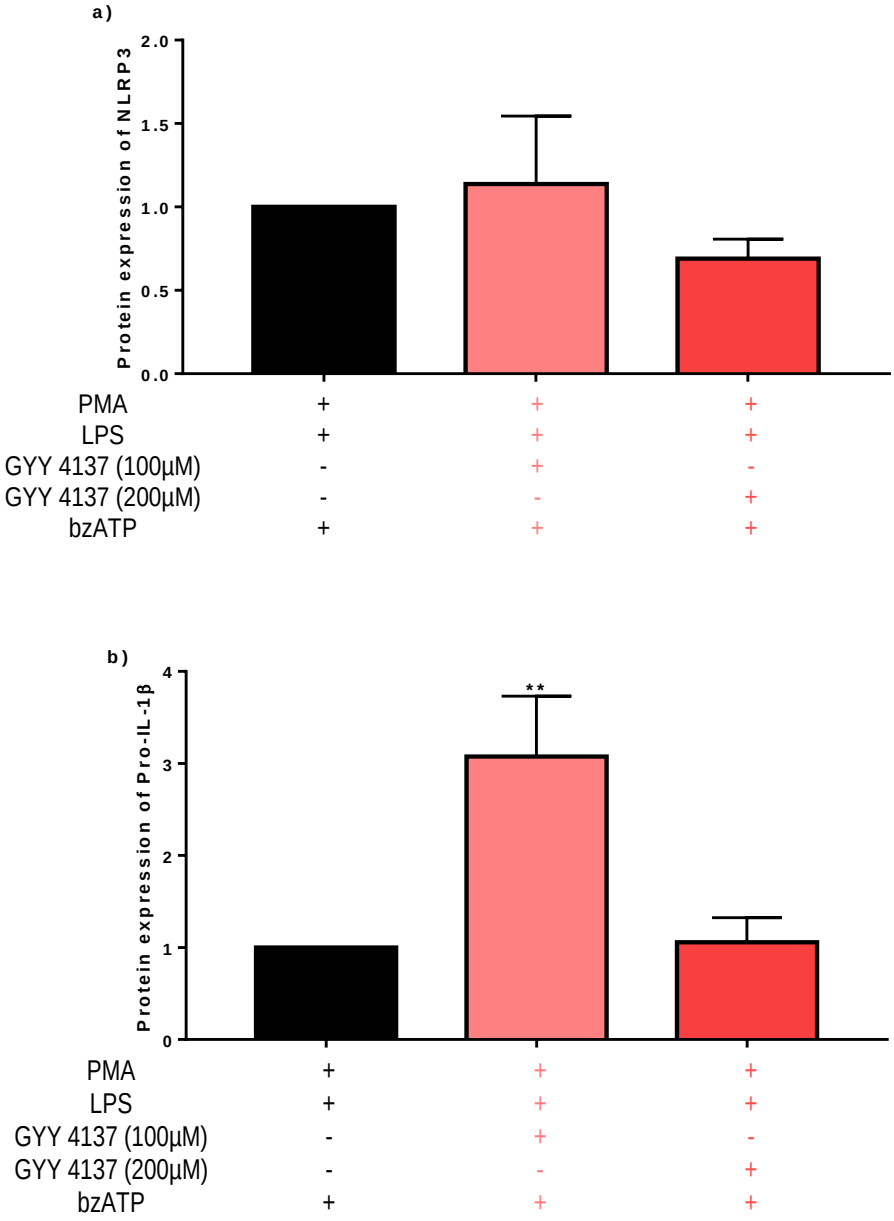

Figure 5-5 bzATP effect of GYY4137 on NLRP3 inflammasome Signal 1 in THP-1 cells.

Western blot bands for NLRP3 (**a**) and pro-IL-1β (**b**) were analysed by densitometry relative to β-actin housekeeper. GYY4137 (100µM) increased pro-IL-1β (**p<0.01 compared to PMA+LPS+bzATP treatment). Data presented as mean ±SEM, n=3. Statistical analysis (ANOVA) was used to compare treated cells with PMA+LPS treatment.

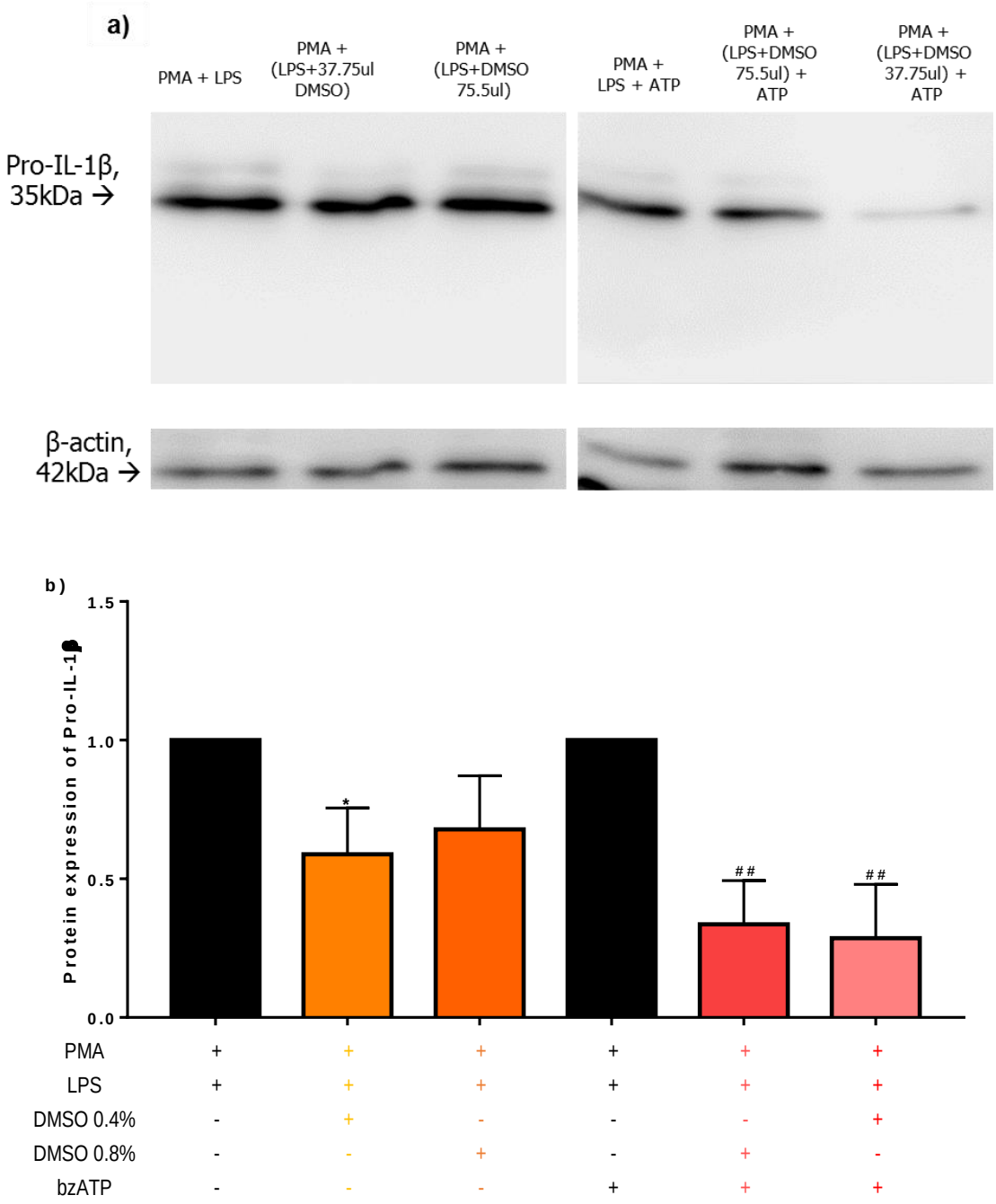

Figure 5-6 Effect of DMSO on NLRP3 inflammasome Signal 1 in differentiated THP-1 cells.

Western blot bands (**a**) for pro-IL-1β was analysed by densitometry relative to β-actin housekeeper. GYY4137 reduced pro-IL-1β (*$p<0.05$ and $^{\#\#}p<0.01$ compared to PMA+LPS and PMA+LPS+bzATP treatment, respectively) by ANOVA (**b**). Data presented as mean ± SEM, n=3.

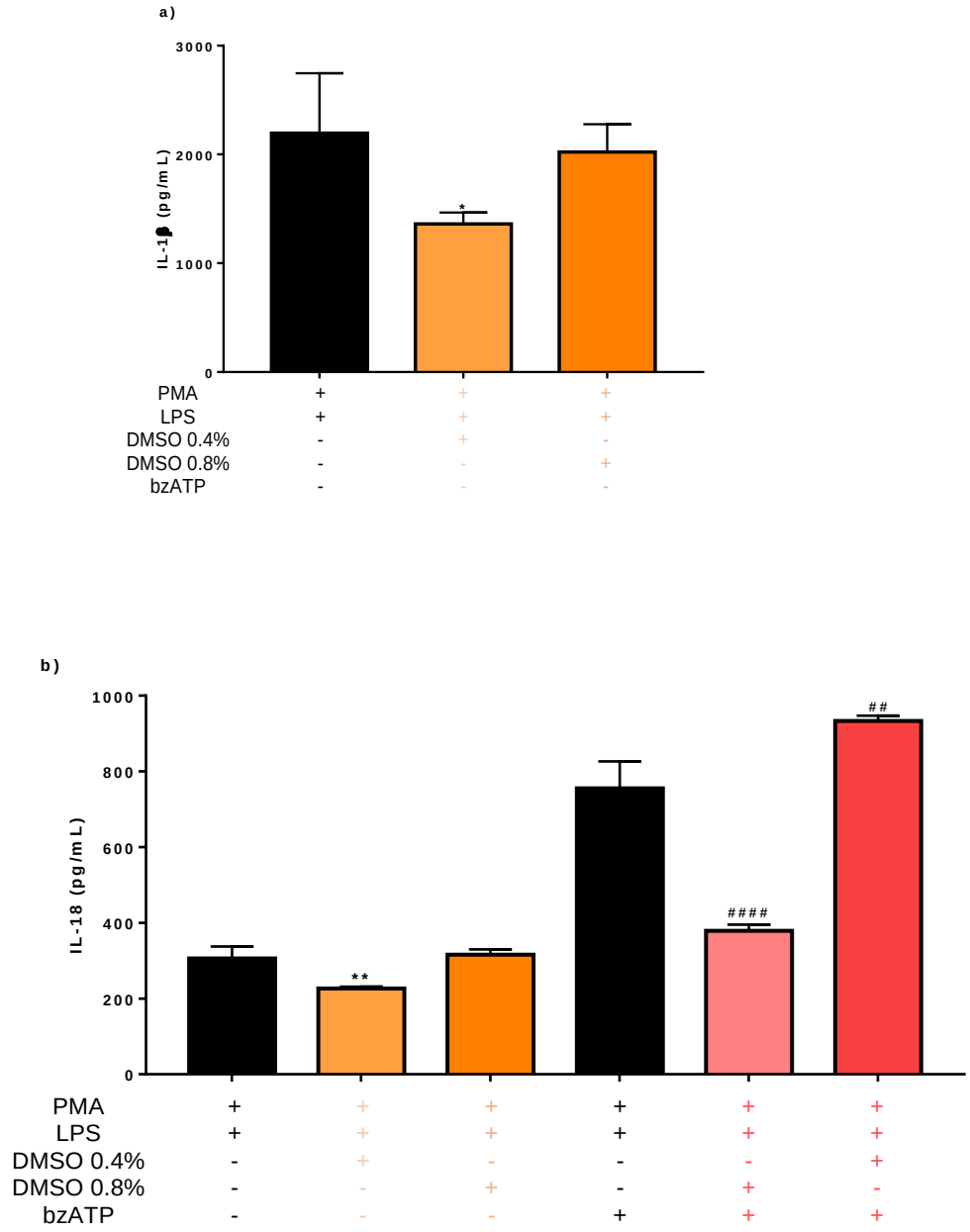

Figure 5-7 Effect of DMSO on NLRP3 inflammasome Signal 2 in differentiated THP-1 cells.

IL-1β and IL-18 were measured by ELISA in cell culture media following DMSO treatment. IL-1β was significantly reduced with low concentration (0.4%) of DMSO (*p<0.05 compared to PMA+LPS treatment) by ANOVA (**a**). IL-18 level was significantly affected by DMSO (**p<0.01 compared to PMA+LPS and $^{\#\#}$p<0.01, $^{\#\#\#\#}$p<0.0001 compared to PMA+LPS+bzATP treatment), by ANOVA (**b**). Data presented as mean ± SEM, n=3.

5.4.2 Development of a method for quantitation of NLRP3 gene expression by qPCR

As part of the Signal 1 NLRP3 inflammasome pathway involves transcription and/or translational upregulation of constituent proteins, the gene expression level of NLRP3 protein was further investigated transcriptionally by qPCR (Bostanci et al., 2011). Western blot analysis of NLRP3 was not optimal, possibly due to the primary antibody, and an alternative assessment of NLRP3 gene expression was investigated.

5.4.2.1 Primer design

Selection of housekeeper genes for THP-1 cells was based on a previous study (Maeß et al., 2010). Two most stably expressed genes were identified; *RPL37A* (ribosomal protein L37A) and *ACTB* (β-actin) which were validated by investigating mRNA expression of macrophage scavenger receptor CD36 in PMA treated THP-1 cells.

Subsequently, exons of the gene of interest (*NLRP3*) and both housekeeper genes (*RPL37A* and *ACTB*) were identified via GenBank® (http://www.ncbi.nlm.nih.gov/gene) and confirmed via Ensemble (https://www.ensembl.org/). The range of nucleotide sequence of the gene of interest between bases 1030 to 2902 from 3' end was determined using primer-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) via RefSeq (https://www.ncbi.nlm.nih.gov/refseq/). The list of amplified target specific primers was identified (**Figures 5-8**) and the higher specificity primers were chosen based on the matches closer to the 3' end (Ye et al., 2012). Similarly, the primers were designed for the two housekeeping genes. The parameters in **Table 5-1** were applied to select specific primer pairs.

Parameters	Value
PCR product size	70 base pair to 300 base pair (bp)
# primers to return	10
Primers melting temperature (T_m)	Min 57°C, Opt 61°C and Max 65°C
Exon junction span	Primers must span an exon-exon junction

Table 5-1 Parameters for selection of PCR primers.

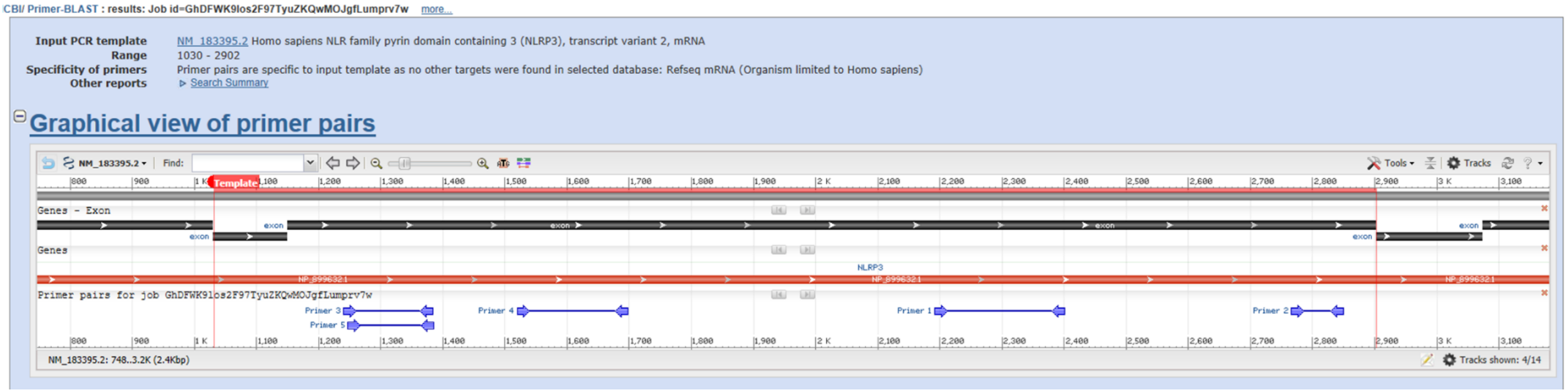

Figure 5-8 Designing the target-specific primers.

Primer-BLAST returned five specific primer pairs (blue) for amplification by qPCR of the 3' end of the NLRP3 coding sequence (red). The detailed alignment between target gene and primer are shown.

As a result, specific primer pairs (**Table 5-2**) were identified for gene of interest and both housekeeper genes:

No	Gene	Primer sequence	Amplicon length (bp)	T_m, °C
1	*NLRP3*	F: CCTGTTTGAGGAGTCCGACC	200	60
		R: TCAAACGACTCCCTGGAACG		
2	*NLRP3*	F: CATCGGGTGGAGTCACTGTC	90	60
		R: AAGGTGTCGGCCTTCCTTTT		
3	*NLRP3*	F: CAAACGCTACACACGACTGC	175	60
		R: CATCGGGGTCAAACAGCAAC		

Table 5-2 Primer sets for *NLRP3.*

No	Gene	Primer sequence	Amplicon length (bp)	T_m, °C
1	*RPL37A*	F: GGCGACATGGCCAAACGTACC	120	62
		R: AGTGTACTTGGCGTGCTGGCT		
2	*RPL37A*	F: CCAAGATGAAGAGACGAGCTG	100	62
		R:TGACAGCGGAAGTGGTATTG		
3	*RPL37A*	F: ACAATACCACTTCCGCTGTC	100	62
		R:CCAGTGATGTCTCAAAGAGTAGAG		
4	*RPL37A*	F: ATTACTTGAGGCCAGGAGATTG	122	62
		R:CCTCCAAAGTAGCTGGGATTAC		
5	*RPL37A*	F: GACCTACACCATCAGCATCTTC	106	62
		R:ACGAATCCTTACGTGCAAACTA		
6	*RPL37A*	F: GGTGATGTAGATAGGGCTGAAG	110	62
		R:GGAAGAGGCAGGTAAGGAATAC		

Table 5-3 Primer sets for *RPL37A*.

No	Gene	Primer sequence	Amplicon length (bp)	T_m, °C
1	*ACTB*	F:GGCGGCGCCCTATAAAACC	134	62
		R:TCATCATCCATGGTGAGCTGGC		
2	*ACTB*	F:TATAAAACCCAGCGGCGCGA	120	62
		R:TCATCCATGGTGAGCTGGCGG		
3	*ACTB*	F:GGATCAGCAAGCAGGAGTATG	100	62
		R:AGAAAGGGTGTAACGCAACTAA		
4	*ACTB*	F:TCCACCGCAAATGCTTCT	100	62
		R:AGCCATGCCAATCTCATCTT		

Table 5-4 Primer sets for *ACTB*.

5.4.2.2 RNA isolation

Five groups of experiments were performed in triplicate (**Figure 5-9**). LPS-treated differentiated THP-1 cells were used as a positive control and compared with DMSO or GYY4137 treated cells.

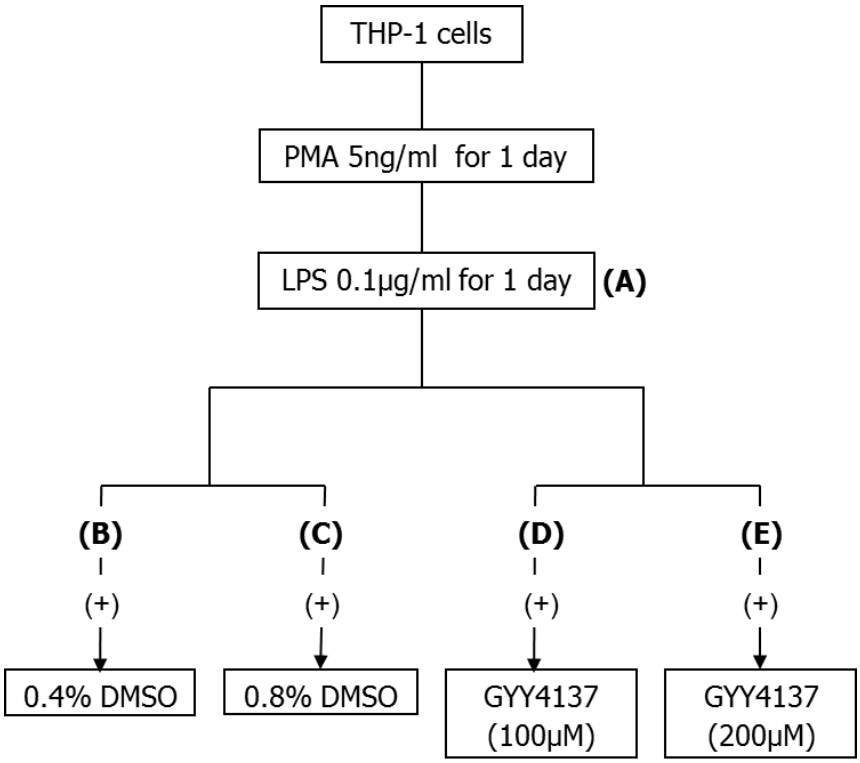

Figure 5-9 Flow chart to investigate the effect of hydrogen sulfide donor GYY4137 on NLRP3 inflammasome activation in THP-1 cells

Flow diagram shows step-by-step treatments of THP-1 cells prior to qPCR analysis.

A NanoDrop microvolume spectrophotometer was used to measure the RNA concentration, in triplicate. RNA concentration ranged from 117.5ng/µl to 663.4ng/µl.

5.4.2.3 cDNA synthesis

Subsequently, all the RNAs were subjected to reverse transcription polymerase chain reaction (RT-PCR) for 30 cycles to synthesize single-stranded cDNA (see Method section, **Table 2-11**). Three set of controls were performed; without RT enzyme, without RNA and water only.

5.4.2.4 Assessment of primers

cDNA from each group of treatment was mixed with each primer set and PCR carried out according to the conditions set out in **Table 5-5** (Vallone and Butler, 2004)**.** Subsequently, these PCR products were analysed by agarose gel electrophoresis to assess for any non-specific priming, primer dimer or hairpin formation (**Figure 5-10**). The appearance of degraded or smeared bands on the agarose gel was considered to be a sign of non-quality primers (Zhang et al., 2004).

Step	Temperature, °C	Duration, sec
Denaturation	94	15
Annealing	62	15
Elongation	72	30

Table 5-5 Temperature settings for PCR.

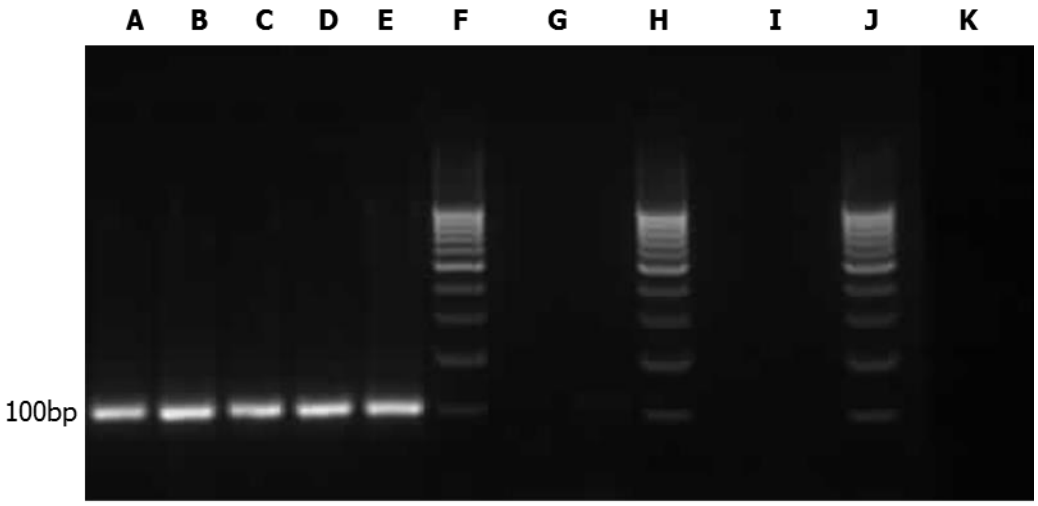

Figure 5-10 Analysis of PCR products by agarose gel electrophoresis.

Example of quality primer with strong bands visualized for primer set two (*NLRP3*). RNA isolated from cells treated with PMA+LPS (**A**), PMA+0.4% DMSO (**B**), PMA+0.8% DMSO (**C**), PMA+LPS+100µM GYY4137 (**D**), PMA+LPS+200µM GYY4137 (**E**), ladder (**F**,**H** and **J**), without RT (**G**), without RNA (**I**) and water only (**K**). Single brightly-stained bands were observed at approximately 100bp for all samples. As expected, no bands were visualised in all three sets of control experiment.

As result, the best primer sets were selected for further qPCR analysis, as in **Table 5-8**:

Primer pair	Sequence
NLRP3	F: CATCGGGTGGAGTCACTGTC
	R: AAGGTGTCGGCCTTCCTTTT
NLRP3	F: CAAACGCTACACACGACTGC
	R: CATCGGGGTCAAACAGCAAC
RPL37A	F: GGCGACATGGCCAAACGTACC
	R: AGTGTACTTGGCGTGCTGGCT
RPL37A	F: ACAATACCACTTCCGCTGTC
	R:CCAGTGATGTCTCAAAGAGTAGAG
ACTB	F:GGATCAGCAAGCAGGAGTATG
	R:AGAAAGGGTGTAACGCAACTAA
ACTB	F:TCCACCGCAAATGCTTCT
	R:AGCCATGCCAATCTCATCTT

Table 5-6 Lists of the best primer pairs for gene of interest and the housekeeper genes.

5.4.2.5 Effect of GYY4137 on NLRP3 mRNA expression

Using qPCR, and an annealing temperature of 60°C no amplification was observed. With an annealing temperature of 62°C excellent amplification was observed (**Figure 5-11**). The quantitative real-time PCR data was analysed by the comparative C_T method as described in Materials and Methods and as mentioned previously (Schmittgen and Livak, 2008).

Even though there was no significant difference between GYY treatment as compared to control (PMA+LPS), there was a trend towards reduced NLRP3 gene expression as compared to corresponding DMSO treatment (**Figure 5-12)**.

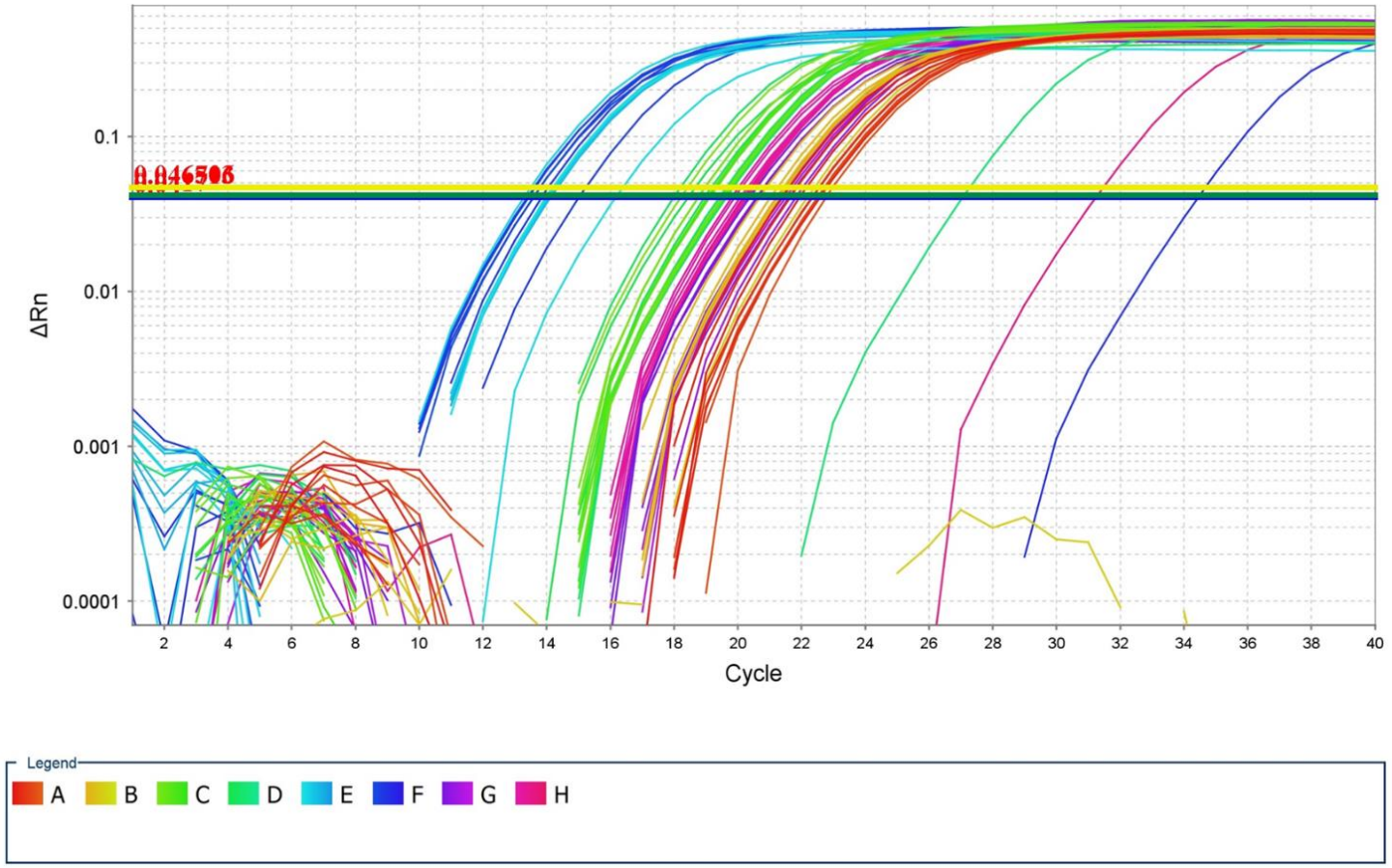

Figure 5-11 Amplification curve of qPCR.

The C_T value was generated from the point where the amplification plot crosses the threshold fluorescence (yellow line). Bottom left plots showed water-only treatment. A,B *NLRP3*; C,D *RPL37A*; E,F *ACTB* and G,H *NLRP3* sample #2.

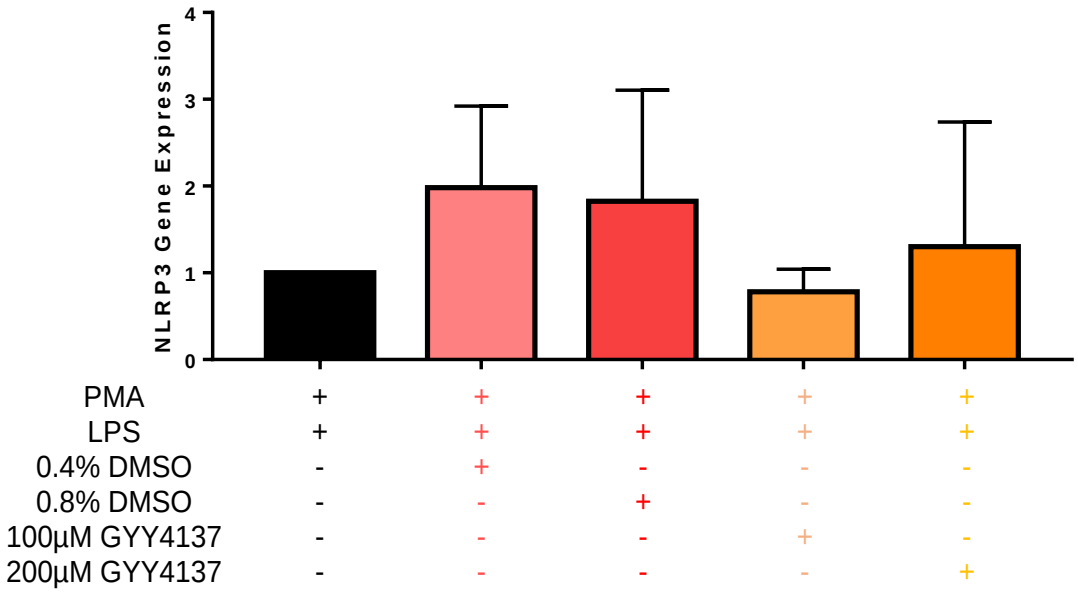

Figure 5-12 Effect of DMSO and DMSO-diluted GYY4137 on NLRP3 expression in LPS-treated differentiated THP-1 cells.

qPCR for NLRP3 gene expression showed no significant difference on each treated cell compared to PMA+LPS group by ANOVA. Data presented as mean ± SEM, n=3.

5.4.3 Effect of GYY4137 on NLRP3 inflammasome activation

Based on previous findings, DMSO was replaced with PBS as diluent for GYY4137. Signal 1 was measured semi-quantitatively using Western blot. Firstly it was noted that GYY4137 could not replace LPS as a Signal 1 agent in differentiated THP-1 cells (**Figure 5-13a** and **Figure 5-13b**). There was a suggestion that GYY4137 was able to increase Signal 1 activation in THP-1 cells as NLRP3 level increased at 200μM GYY4137 (**Figure 5-14a**) and there was a trend for pro-IL-1β level to increase (**Figure 5-14b**).

By ELISA there was a trend in reduction of active IL-1β stimulation with LPS administration at both GYY4137 concentration (**Figure 5-15a**). For IL-18 there was a concentration dependent decrease in synthesis (**Figure 5.15b**) and the difference was statistically significant at both concentrations following bzATP administration.

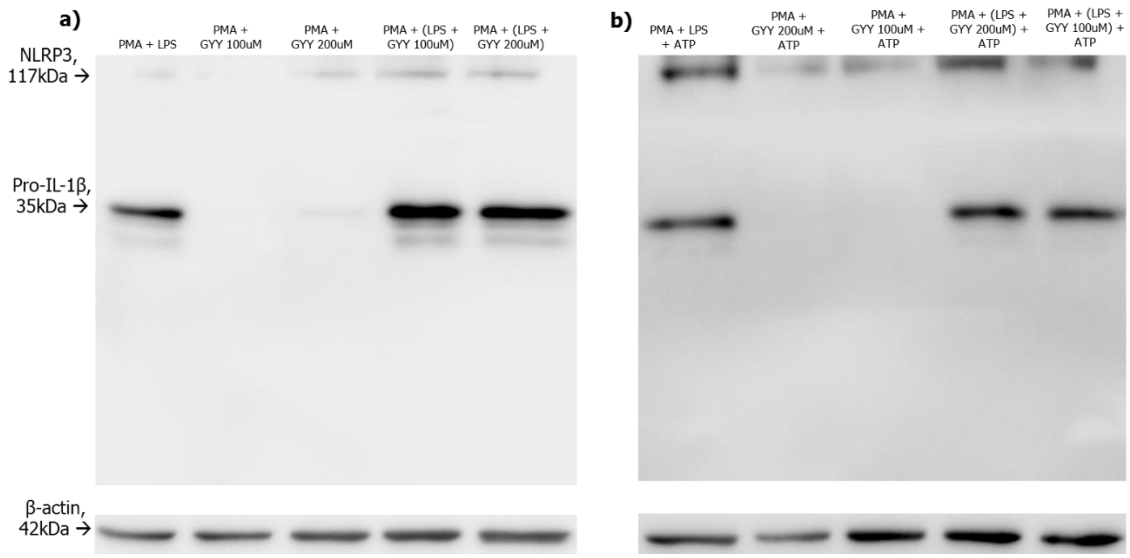

Figure 5-13 Signal 1 expression, NLRP3 and pro-IL-1β on Western blot of differentiated THP-1 cells.

Western blot protein expression of NLRP3 and pro-IL-1β (Signal 1) in 50µg of differentiated THP-1 cell lysates. Differentiated THP-1 cells were treated with or without LPS (**a**). BzATP was added at the end of each treatment (**b**).

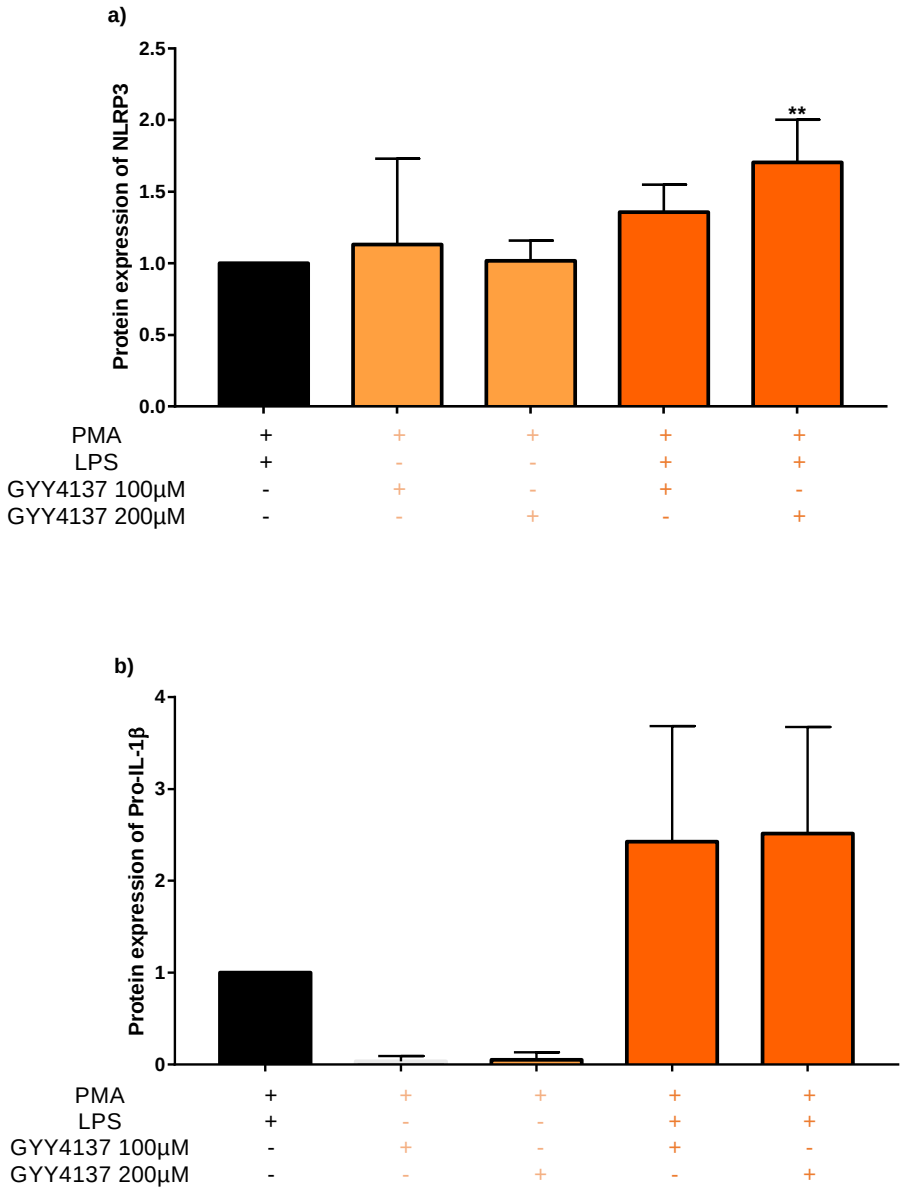

Figure 5-14 Effect of GYY4137 on Signal 1 in differentiated THP-1 cells.

Western blot bands for NLRP3 (**a**) and pro-IL-1β (**b**) were analysed by densitometry relative to β-actin housekeeper. GYY4137 (200µM) increased NLRP3 expression (**p<0.01). Data presented as mean ± SEM, n=3. Statistical analysis (ANOVA) was used to compare treated cells with PMA+LPS control samples.

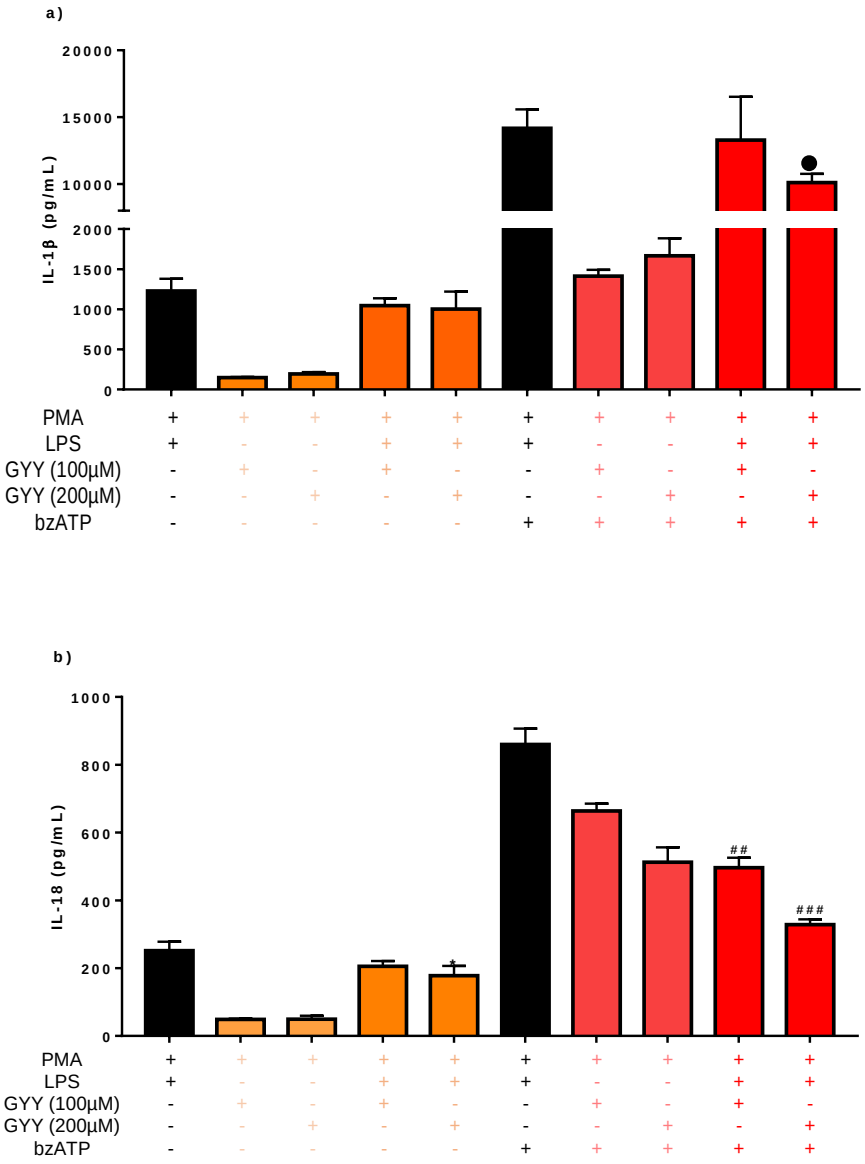

Figure 5-15 Effect of GYY4137 on IL-1β and IL-18 synthesis in differentiated THP-1 cells.

THP-1 cells were differentiated using PMA following by Signal 1 activation by LPS for 24 hours. Subsequently, bzATP was administered to activate Signal 2. Data presented as mean ± SEM, n=3 (*p<0.05 compared to PMA+LPS or $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared to PMA+LPS+bzATP) by ANOVA. •p<0.05 compared to PMA+LPS+bzATP by unpaired student t-test.

5.4.4 Effect of AP39 on Signal 1 and Signal 2 NLRP3 inflammasome activation in THP-1 cells

The effect of mitochondria targeted H_2S donor, AP39, was then investigated in differentiated THP-1 cells. AP39 was added to cell cultures with or without LPS. Signal 1 expression (pro-IL-1β) was measured semi-quantitatively from the Western blot (**Figure 5.16a**). Two concentrations of AP39 were used in this experiment, 100nM and 300nM based on previous studies (Szczesny et al., 2014).

AP39 was unable to trigger pro-IL-1β expression in differentiated THP-1 cells, compared to LPS. Moreover, there was no evidence that AP39 was able to reduce NLRP3 inflammasome Signal 1 as pro-IL-1β levels induced by LPS, were not affected (**Figure 5-16b**).

Further investigation with ELISA revealed that both concentrations of AP39 manifest different inflammatory properties in IL-1β expression of differentiated THP-1 cells with bzATP administration. Interestingly, at the lower concentration of AP39 (100nM), AP39 potentiated IL-1β concentration by 3 fold (**Figure 5-17a**). However, in the treatment without bzATP administration, both concentrations of AP39 significantly increased IL-18 expression. However, both concentrations of AP39 significantly decreased IL-18 expression with bzATP administration suggesting an anti-inflammatory effect (**Figure 5-17b**).

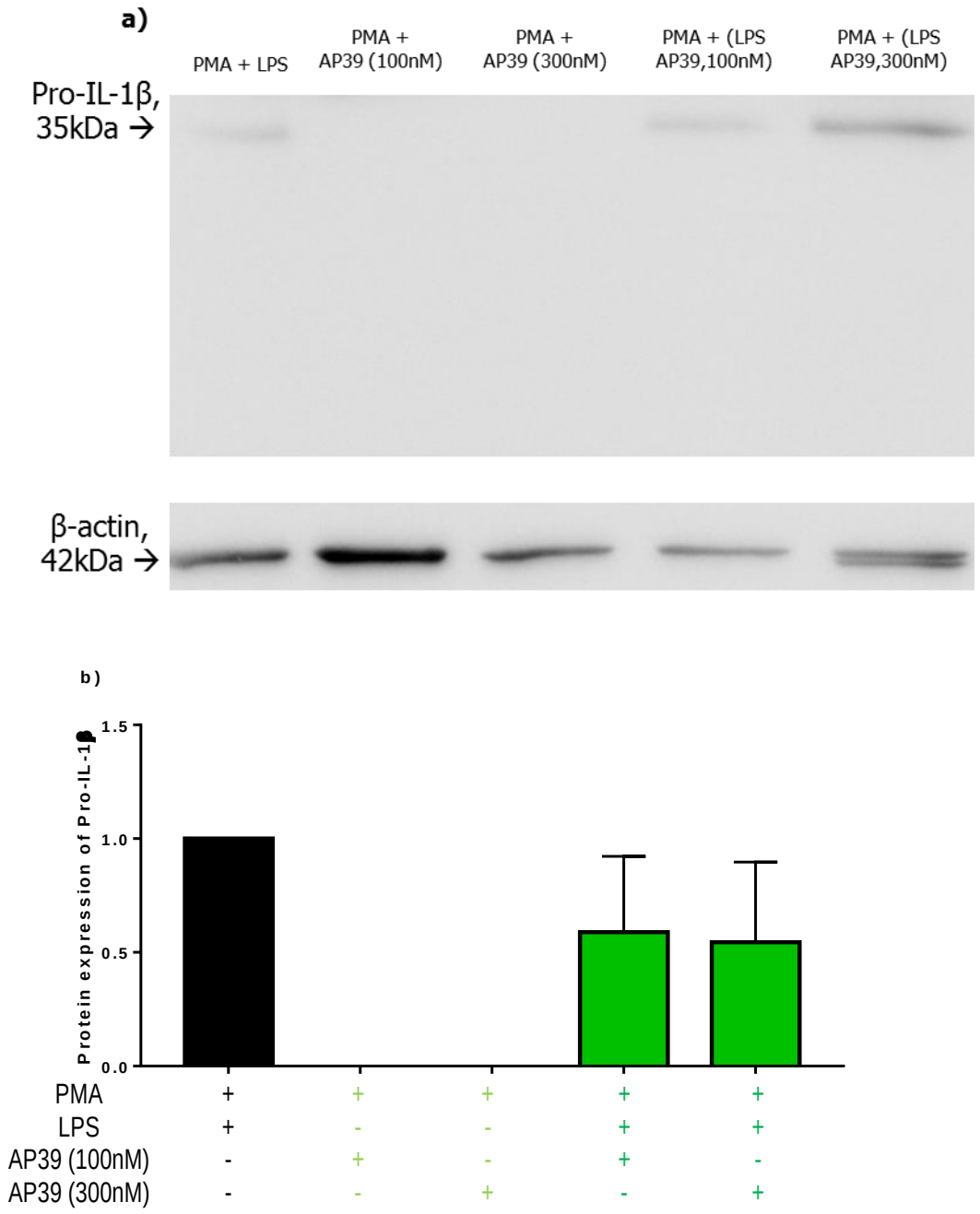

Figure 5-16 Effect of AP39 on Signal 1 in differentiated THP-1 cells.

Differentiated THP-1+LPS were treated with AP39 for 24 hours and lysates formed. Lysates were analysed by Western blot. Pro-IL-1β band (**a**) were analysed by densitometry (**b**) relative to β-actin housekeeper. Data presented as mean ± SEM, n=3.

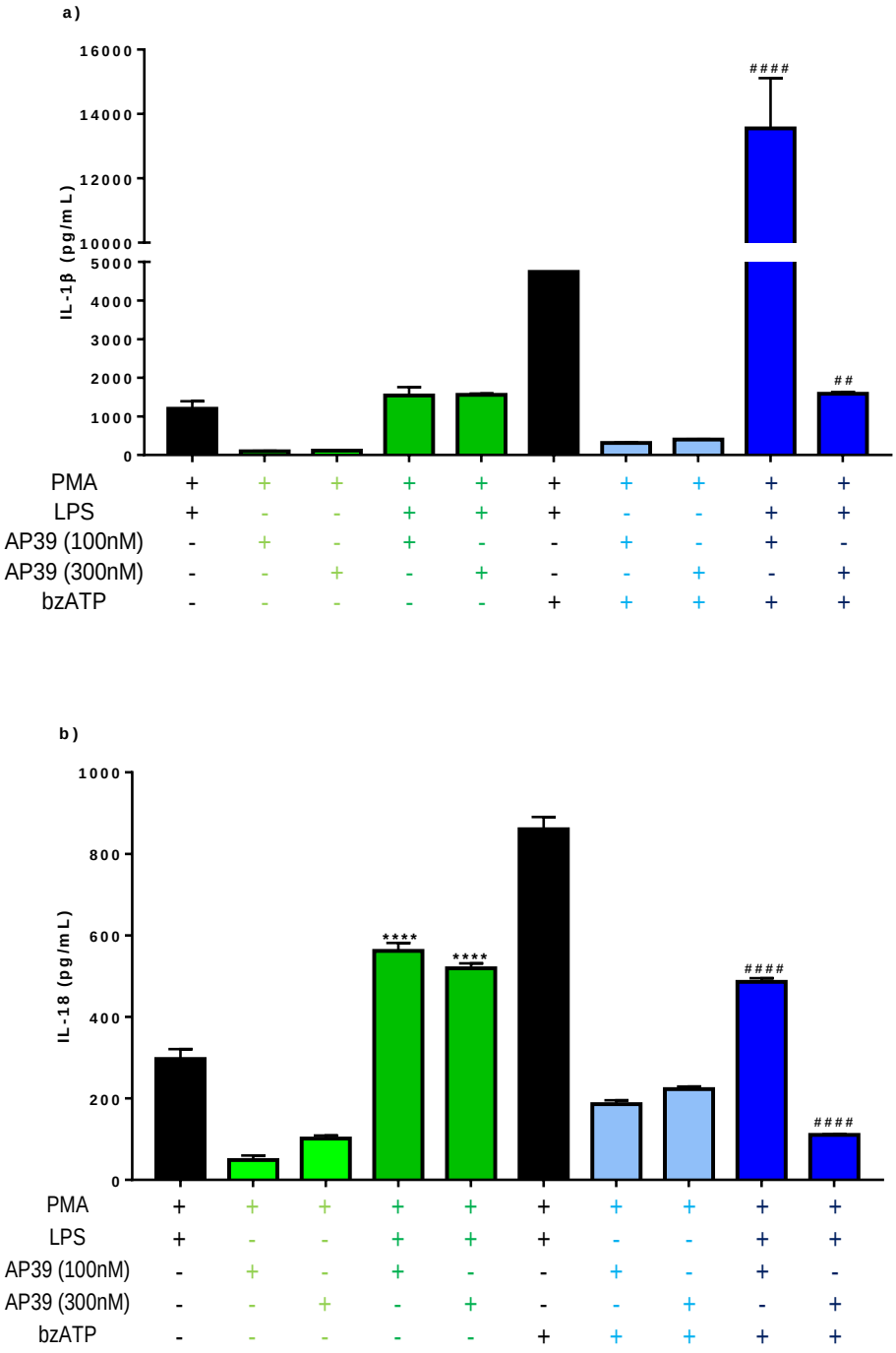

Figure 5-17 Effect of AP39 on IL-1β and IL-18 synthesis in differentiated THP-1 cells.

THP-1 cells were differentiated using PMA following by Signal 1 activation by LPS for 24 hours. Subsequently, bzATP was administered to activate Signal 2. Data presented as mean ± SEM, n=3 (****p<0.0001 compared to PMA+LPS or ##p<0.01 and ####p<0.0001 compared to PMA+LPS+bzATP) by ANOVA.

5.5 Discussion

5.5.1 Hydrogen sulfide and NLRP3 inflammasome priming (Signal 1)

In this chapter, the role of exogenous H_2S in NLRP3 inflammasome activation was studied in THP-1 cells. Signal 1 inflammasome activation was investigated by Western blot of NLRP3 and pro-IL-1β and Signal 2 inflammasome activation was measured by active IL-1β and active IL-18 by ELISA.

This study focused on slow-releasing hydrogen sulfide donors in view of their stability and as slow-release is a good model for endogenous H_2S biosynthesis. Two concentrations were used; 100µM and 200µM of GYY4137 as mentioned previously (Whiteman et al., 2010). However, in that study RAW 264.7 macrophages were used instead of THP-1 cells which might affect the NLRP3 inflammasome, evidenced by less ASC-speck like formation (Bryan et al., 2010). Furthermore, previous studies have not focused specifically on the Signal 1 component of the NLRP3 inflammasome.

Reduced pro-IL-1β production suggested that the hydrogen sulfide donor reduced Signal 1 or priming which might be related with reduced NF-κB activation (Oh et al., 2006b). Furthermore, this study also mentioned that the pre-incubation treatment with hydrogen sulfide donors in LPS-treated macrophages inhibited nitric oxide and iNOS production via heme oxygenase and carbon monoxide action, which might be of benefit in future studies.

Interestingly, a pro-inflammatory effect such as the increased pro-IL-1β production by the lower concentration of GYY4137 treatment might be explained by a previous study mentioning that a H_2S donor could mimick the K_{ATP} channel, hence mediated the inflammatory process (Zanardo et al., 2006). Furthermore, bzATP administration enhanced the activation of K_{ATP} channel (Juliana et al., 2012). Further treatment with ATP degrading enzyme, apyrase, before LPS administration might be useful in investigating the expression of pro-IL-1β either directly by bzATP or by exogenous hydrogen sulfide (Grahames et al., 1999).

Even though the results presented in this chapter suggested anti-inflammatory effects (reduced Signal 1) of GYY4137, DMSO itself has been proved previously for its anti-inflammasome property by hydroxyl radical scavenger activity (Colucci et al., 2008).

Indeed and interestingly, results showed significantly reduced pro-IL-1β production (Signal 1) in DMSO-only treated LPS-differentiated THP-1 cells. This discovery disproved the previous finding mentioning that inflammasome production was only disrupted if the final concentration of DMSO was more than 1%, as in this thesis the DMSO concentration was 0.8% (Ahn et al., 2014). The mechanism is thought to be via transcriptional downregulation of NF-κB associated with decreased mRNA secretion of pro-inflammatory cytokines (Hollebeeck et al., 2011). However, that study was focused mainly on Signal 2 inflammasome rather than priming effect (Signal 1).

Further investigation with ELISA confirmed the reduction of Signal 2 NLRP3 inflammasome (IL-1β and IL-18) even with as little as 0.4% DMSO, especially for IL-18 production. Non-caspase proteases or proteinase 3 (PR3) also might be stimulated to cleave pro-IL-18 to bioactive form of active IL-18 (Van de Veerdonk et al., 2011) in an inflammasome-independent pathway. However, no changes of active IL-1β production were observed following bzATP suggesting DMSO might act via caspase-1 activation pathway (Luheshi et al., 2012).

Additionally, NLRP3 gene expression can be modulated transcriptionally as well as post-transcriptionally by certain signalling receptors (Bauernfeind et al., 2009). Even though there was no relationship between GYY4137 and Signal 1 gene expression transcriptionally, this could be explained by a previous study mentioning the signalling by TLR4 through MyD88 could rapidly and non-transcriptionally prime NLRP3 by stimulating its deubiquitination (Juliana et al., 2012). Further investigation of bzATP administration after GYY4137 treatment might be helpful in investigating the relationship between mtROS production by bzATP and transcriptional induction of NLRP3 (Bauernfeind et al., 2011).

Subsequently DMSO-diluted GYY4137 was replaced with PBS-diluted GYY4137 in LPS-treated differentiated THP-1 cells, with and without bzATP. Additionally, GYY4137-only treatment also was introduced in differentiated THP-1 cells to study its effect in Signal 1 production, that is, could it replace LPS function.

In the treatment without bzATP, results showed slight but significant increased NLRP3 protein expression for higher concentrations of GYY4137. However, no significant increment was observed in pro-IL-1β protein expression. This finding suggested a hydrogen sulfide donor was able to increase Signal 1 or priming of NLRP3 inflammasome in differentiated THP-1 cells also primed with LPS. Adding hydrogen sulfide donor possibly enhanced the accumulation of Signal 1 end product via NF-κB activation. (Huang et al., 2016). Further investigation on pro-caspase-1 expression might be helpful to investigate the inflammasome pathway further (Mariathasan et al., 2004). These data also showed that GYY4137 alone was not able to act as a Signal 1 inducer, that is, it could not replace LPS.

Subsequently, treatment with AP39 revealed that this slow-released H_2S donor was unable to replace LPS as Signal 1 inducing agent of the NLRP3 inflammasome. This finding could be explained as NF-κB activation is required for priming of NLRP3 inflammasome whilst AP39 preserved ATP levels, decreased mitochondrial oxidant production and hence acts as cytoprotective (Le Trionnaire et al., 2014). That study revealed that AP39 was able to inhibit oxidative stress in the mitochondria of THP-1 cells. Further investigation of mtROS production might be helpful to delineate the potential mechanism of non-transcriptional Signal 1 inflammasome inhibition by AP39 treatment (Juliana et al., 2012).

5.5.2 Hydrogen sulfide and NLRP3 inflammasome activation (Signal 2)

Exogenous hydrogen sulfide (NaHS) was shown to decrease NLRP3 inflammasome Signal 2 activation by suppressing the NF-κB pathway (Huang et al., 2016). Interestingly, further investigation revealed that a slow-releasing H_2S donor such as GYY4137 showed compelling mtROS inhibition in professional immune cells hence reduced mature IL-1β production via blockage of caspase-1 and ASC oligomerization (Castelblanco et al., 2017).

In this study, we aimed to investigate the effect of slow-releasing H_2S donor, GYY4137 and mitochondrial-targeted H_2S donor, AP39 towards the Signal 2 end products of NLRP3 inflammasome; IL-1β and IL-18 in differentiated THP-1 cells.

As expected, IL-1β expression was much higher in all treated and untreated differentiated THP-1 cells as compared to IL-18, with or without bzATP. A previous study showed that IL-1β is one of the most potent pro-inflammatory cytokines and shown to be more protective in several bacterial infections (Sahoo et al., 2011). Moreover, it was well documented that ROS homeostasis is more related to the Signal 1 process of pro-IL-1β rather than pro-IL-18 by transcriptional aggravation (Bauernfeind et al., 2011). Additionally, IL-18 also stimulates other cytokine productions such as TNFα, IL-1β and IL-8 (Sahoo et al., 2011).

We also investigated Signal 2 in non-primed differentiated THP-1 cells. GYY4137 was added in differentiated THP-1 cells, replacing LPS function. Results showed that both concentrations of GYY4137 showed a limited ability to promote IL-1β and IL-18, suggesting GYY4137 is not a Signal 2 activation agent. These findings support previous studies where this slow-releasing H_2S donor was unable to promote inflammation in intact cells (Li et al., 2013a).

Subsequent treatment with bzATP revealed that both concentrations of GYY4137 were able to act in an anti-inflammatory way in LPS-treated differentiated THP-1 cells, especially in IL-18 released inhibition.

It was observed that the higher concentration of GYY4137 was able to reduce IL-1β released in LPS-treated differentiated THP-1 cells. This predicted finding was

supported by a previous study (Whiteman et al., 2010). Higher concentration of LPS or prolonged LPS treatment of the same concentration might be useful for further investigation of the effect of GYY4137 on IL-1β (El-Senduny and Bilitewski, 2013).

Subsequently, GYY4137 was replaced with AP39 in the same experimental model. AP39 is believed to act as an exogenous slow-releasing H_2S donor specifically in mitochondria (Wood et al., 2013). Two concentrations were used in this experiment; 100nM and 300nM as mentioned previously (Szczesny et al., 2014).

In the treatments without bzATP administration, both concentrations of AP39 significantly increased IL-18 production, suggesting a pro-inflammatory effect and possible direct Signal 2 effect. These results suggest AP39 concentration acts as Signal 2 activator in LPS-primed differentiated THP-1 cells partly replacing bzATP function. One limitation of this study is that a control compound that targets mitochondria but does not release H2S is required. Le Trionnaire et al. (2014) described TPP^+ derivative of dithiolethione as such a control. It might be that disturbance of mitochondrial membrane potential has an effect on NLRP3 inflammasome activation.

Like GYY4137, treatment with AP39 showed significant anti-inflammatory effect especially in IL-18 production in the presence of bzATP but also at 300nM AP39 for IL-1β synthesis. A previous study mentioned that AP39 attenuated pro-inflammatory phenotype in an epithelial cell model using glucose oxidase (GOx)-induced oxidative stress by decreased intracellular oxidant content and reduced necrosis (Ahmad et al., 2016).

Conversely, 100nM of AP39 showed significant increased of IL-1β indicating a pro-inflammatory effect in the presence of bzATP. This finding suggested that low concentrations of AP39 are unable to increase H_2S level within the mitochondrial compartment hence incapable to maintain mitochondrial DNA integrity as well as its function, resulting in pro-inflammatory cytokine (IL-1β) released (Ahmad et al., 2016). Perhaps, comparison with AP219 might be useful as a control compound

for AP39, which lacking the H_2S-releasing portion. Studies of the non-canonical inflammasome pathway should be carried out to further investigate the role of AP39 in Signal 2 process of the NLRP3 inflammasome in view of the discrepancy of the cytokine end products.

5.6 Conclusions

It can be concluded that, DMSO is masking the Signal 1 and Signal 2 anti-inflammasome effect of GYY4137. Therefore, the subsequent experiments were performed with PBS-diluted GYY4137 or DMSO vehicle control should always be used. The results of this chapter can be summarized as shown in **Table 5-7.**

		Signal 1	Signal 2
GYY4137	100µM	NS	(-) (IL-18)
		NS (mRNA)	
	200µM	(+) (NLRP3 only)	(-) (IL-1β and lL-18)
		NS (mRNA)	
AP39	100nM	NS	(+) (IL-1β)
			(-) (IL-18)
	300nM	NS	(-) (IL-1β and lL-18)

Table 5-7 Effect of H_2S donors on NLRP3 inflammasome activation in THP-1 cells.

In summary, no evidence of Signal 1 activation or inhibition by hydrogen sulfide donors was observed either transcriptionally or translationally. Perhaps, more experiments should be carried out to increase “n” number. Both high concentration of GYY4137 and AP39 were proved to inhibit Signal 2 NLRP3 inflammasome in LPS-treated, differentiated THP-1 cells. In contrast and interestingly, a low concentration of AP39 behaved as pro-inflammatory as it increased IL-1β production.

Chapter 6 General discussion

6.1 Signal 1 and Signal 2 NLRP3 inflammasome in THP-1 cells

Even though the THP-1 cell has been intensively used in *in vitro* models of macrophages (Park et al., 2007), there are a few other monocytic cell lines that are commonly used in biomedical research including U937, ML-2, HL-60 and Mono Mac 6 cells (Chanput et al., 2014). However, THP-1 cells have more advantages in term of rapid differentiation and response to LPS treatment (Sharif et al., 2007). Other than PMA, 1,25-dihydroxyvitamin D3 (VD3) is known as one of the THP differentiation agents, however VD3 showed less PKC translocation and TNF-α production (Schwende et al., 1996b).

To the best of our knowledge, peripheral blood mononuclear cell-derived macrophages show a similar response to THP-1 cells in terms of the phagocytic activity and the production of IL-1β and TNF-α after treatment with PMA (Daigneault et al., 2010). Due to uniform genetic background, further advantages of using THP-1 cells include high growth rate, immortalized cells, stable storage in liquid nitrogen and less contamination from blood components (Chanput et al., 2014). Additionally, invasive procedures must be carried out to obtain PBMC from human blood (Gordon et al., 2000). However, investigations of higher concentrations of LPS (up to 100ng/ml) or for a longer duration (3 hours) indicate PBMC are worthy of study in view of the greater expression of inflammation-cytokines such as TNF-α, IL-6, IL-8 and IL-10 (Schildberger et al., 2013).

Our study proved that differentiated THP-1 cells exhibit NLRP3 inflammasome activation upon stimulation with LPS and bzATP. However, there is a paucity of studies demonstrating the expression of Signal 1 inflammasome. Signal 1 or priming from the PAMPs or DAMPs is essential to assemble the inflammasome complex. We also had demonstrated that untreated THP-1 cell were unable to produce Signal 1 suggesting CD14 and TLR is not upregulated without THP-1 differentiation (Henning et al., 2008).

Signal 2 or the activation step of the NLRP3 inflammasome requires certain stimulators such as extracellular ATP, hyaluronan, glucose, MSU and Amyloid-β

via induction of caspase activity (Tschopp and Schroder, 2010). Our study proved that extracellular ATP (bzATP), increased IL-1β and IL-18 release into the supernatant. This is reported to be by the process of potassium efflux through $P2X_7$ receptor pore formation (Couillin et al., 2013). Interestingly, a low potassium level in the cytoplasm also triggers the NLRP3 inflammasome activation by apoptosis (Cain et al., 2001). Hence, further investigation of potassium channel blockers might be useful to delineate the exact potassium level that safeguards the integrity of THP-1 cells.

Diversely, other environmental irritants such as silica, asbestos and alum might be used to investigate whether reactive oxygen species are involved in inflammasome pathways other than potassium channel, $P2X_7$. For example, IL-33 a member of IL-1 family is triggered by alum which is strongly dependent on ASC and NLRP3 protein (Li et al., 2008a). Cathepsin B, released from lysosomes by silica also might be worthy of investigation for release of IL-1β in THP-1 cells (Hornung et al., 2008).

6.2 Intracellular superoxide and NLRP3 inflammasome in differentiated THP-1 cells

We used paraquat (PQ) and mitoparaquat (mitoPQ) to produce mitochondrial and cytoplasmic ROS for NLRP3 inflammasome stimulation (Robb et al., 2015). MitoPQ is a novel mitochondria-targeted compound interacting with Complex 1 of respiratory chain hence producing superoxide (Mulvey et al., 2017). Theoretically, this ROS production leads to the production of ROS-induced ROS release (RIRR) hence triggering the neighbouring mitochondrial ROS (Zorov et al., 2000). However, neither PQ nor mitoPQ demonstrated inflammasome activation in differentiated THP-1 cells. A study showed that superoxide dismutase (SOD) protects against redox cycling in mitochondria and might be helpful in further investigations (Cochemé and Murphy, 2008). The data in this thesis obtained in THP-1 cells *in vitro*, agrees with study in Wistar rats (Robb et al., 2015).

Our results showed that intracellular superoxide was unable to replace LPS and did not affect Signal 1 expression in differentiated THP-1 cells prior to LPS

treatment. This is of interest bearing in mind that there are limited studies demonstrating the link between Signal 1 inflammasome and intracellular superoxide. Paraquat was shown previously to increase NLRP3 inflammasome activation in Wistar rat kidney (Liu et al., 2017). However, this paper showed equivocal evidence that Signal 1 was affected by PQ.

Furthermore, our study showed that intracellular superoxide generators were unable to increase pro-caspase-1 expression in LPS-treated differentiated THP-1 cells. A previous *in vivo* study used longer exposure to superoxide generators (72 hours) resulting in increased pro-caspase-1 production (Liu et al., 2015). Further investigation using the caspase-1 inhibitor, Ac-YVAD-CHO might be helpful in investigating caspase-1 related inflammasome production (Ghayur et al., 1997).

Interestingly, a higher concentration of mitoPQ (5μM) was able to increase the Signal 2 NLRP3 inflammasome. MitoPQ is several hundred-fold more potent at producing superoxide as compared to PQ at the flavin site of Complex I in mitochondria (Cochemé and Murphy, 2008). Furthermore, research has demonstrated that mitochondrial oxidative stress was able to trigger the dissociation of thioredoxin-interacting protein (TXNIP) from thioredoxin (Trx) in a ROS-sensitive manner and allowed it to bind to NLRP3, activating the inflammasome (Zhou et al., 2010). The Trx/TXNIP complex plays an important role in regulating cellular redox status and NLRP3 inflammasome activation (Liu et al., 2018). It is worth investigating whether mitochondrial disruption, for example via uptake of mitoPQ, results in mtDNA release and NLRP3 inflammasome activation.

It is hypothesized that mitochondrial superoxide generation is a major cause of degradative diseases and aging via cellular oxidative damage (Darley-Usmar, 2004). Furthermore, lowered antioxidant systems such as SOD, catalase and peroxiredoxin initiate lipid peroxidation cascades to aggravate oxidative stress (Sohal and Weindruch, 1996). There are many related pathologies that are hypothesized to involve oxidative stress in their pathogenesis including inflammation, atherosclerosis, hypertension, cystic fibrosis, diabetes, Parkinson's

disease and Alzheimer's disease (Halliwell and Gutteridge, 1999). Interestingly, mitochondrial calcium uptake also plays a major role in cell signalling and in the regulation of mitochondrial function, which might be beneficial for our future study especially the priming step of NLRP3 inflammasome (Duchen, 2004).

Even though our study demonstrated the stimulation of Signal 2 inflammasome by mitoPQ, the effect might be not be due to direct redox cycler effect. Furthermore, the tested concentration might not be high enough to trigger compromise of the mitochondrial membrane potential (Ross et al., 2005).

TPPB (2-[2-(triphenylphosphonio)ethyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1benzopyran-6-ol bromide) has direct mitochondrial antioxidant effects (Smith et al., 1999). TPPB is believed to be a novel mitochondrial targeted antioxidant, especially in cancer treatment, and was synthesized by attaching the antioxidant phenolic moiety of vitamin E to the lipophilic cation triphenylphosphonium. Further treatment with TPPB in mitoPQ-treated cells would indicate the exact location of ROS generation, either within mitochondria or not (Coulter et al., 2000).

6.3 Hydrogen sulfide donors and NLRP3 inflammasome activation in differentiated THP-1 cells

We further investigated the role of intracellular H_2S release on NLRP3 inflammasome activation using water soluble donors; GYY4137 and AP39. A pioneering study in 2008 proved that GYY4137, a slow-releasing H_2S donor has anti-hypertensive and vasodilator properties without any cytotoxicity effects (Li et al., 2008b).

Our study demonstrated that GYY4137 was unable to act as a priming agent in differentiated THP-1 cells. This is not surprising.

A previous study showed that a H_2S donor reduced NF-κB activation hence reduced the pro-inflammatory cytokines released (Lohninger et al., 2015). Dysregulation of NF-κB triggers the p38 MAPK activation hence leading to inflammation and apoptosis. Further investigation of p38 inhibitors might be useful to study the relationship of H_2S donors on NF-κB priming in differentiated THP-1 cells (Zhi et al., 2007).

Further investigation revealed that GYY4137 increased Signal 1 inflammasome (NLRP3 expression) prior to LPS administration. However, the expression of the *NLRP3* gene was not increased by qPCR. This could be explained by a previous study mentioning the signalling of NLRP3 by TLR4 through MyD88 could rapidly and non-transcriptionally prime the NLRP3 by stimulating its deubiquitination (Juliana et al., 2012). Furthermore, pre-existing cellular NLRP3 inflammasome components might undergo Signal 1 inflammasome activation induced by some priming agents.

There are a paucity of studies demonstrating the effect of H_2S donors on Signal 2 inflammasome. Our results showed reduced IL-1β and IL-18 at both concentrations of GYY4137 tested. These data tally with a study which indicated inhibition of IL-1β and IL-18 production, hence suggesting such compounds act as anti-inflammatory agents as suggested previously (Whiteman et al., 2010). However, that study involved murine RAW264.7 macrophages rather than THP-1 cells which also release other pro-inflammatory cytokines such as NO, TNF-α

and PGE_2 which might be useful in our future studies. However, differences of innate immune response between murine and man are well documented, such as higher NLR expression in human cells (Mestas and Hughes, 2004a). Therefore, this thesis has shown evidence that H_2S is able to inhibit NLRP3 activation in cells with relatively high expression of NLR. This supports the idea that H_2S donor could be potential anti-inflammatory drugs in humans.

A further molecular biology study involving the simultaneous treatment with LPS and a H_2S donor revealed H_2S inhibits histone acetylation and inhibits chromatin openness in THP-1 cells. Subsequently, this chromatin unfolding leads to a decrease in the gene transcription of various pro-inflammatory cytokines (Rios et al., 2015). However, that study used rapid-releasing H_2S, NaHS rather than GYY4137. Measurement of histone deacetylases (HDAC) and histone acetyltransferases (HATs) activities by GYY4137 in THP-1 cell might be useful for further gene regulation study in inflammasome activation (Fuks et al., 2003).

Additionally, further study of foam cell formation in RAW 264.7 macrophages revealed that H_2S suppressed foam cell formation. In future translational research, studying the possible beneficial effects of H_2S donors on atherogenesis, it might be useful to include biomarkers of atherosclerotic risk, such as oxidized LDL (Lynn and Austin, 2011).

AP39 was introduced four years ago as a mitochondria-targeted H_2S donor (Szczesny et al., 2014). However, there are no reported studies on the effect of AP39 towards NLRP3 inflammasome activation.

As predicted, we demonstrated that AP39 acts as an anti-inflammatory agent as it was able to reduce Signal 2 inflammasome activation. Previous study in isolated mouse heart mitochondria demonstrated that AP39 increased Ca^{2+} retention capacity by direct inhibitory effect on mPTP (mitochondrial permeability transition pore) opening, hence manifesting protective effect on ischaemia-reperfusion injury which might be beneficial in our future translational research (Alma, 2016). Additionally, study in a hyperglycaemic model showed extended duration of AP39 exposure restores the mitochondrial membrane potential by an anti-oxidant effect which might be beneficial in a future study (Gero et al., 2016).

Even though previous literature reported the positive and negative effects on inflammation activation (Whiteman and Winyard, 2011), our study has demonstrated the beneficial effect of both GYY4137 and AP39 in reducing the pro-inflammatory cytokines. Limited studies have demonstrated the effect of exogenous H_2S in human cells. Study in human vascular smooth muscle cells showed that exogenous H_2S prevents (NADPH oxidase) NOX-driven intravascular oxidative stress (Muzaffar et al., 2008). Downregulation of NOX-1 protein expression is mediated by the activation of the adenylyl cyclase-cAMP-protein-kinase-G system. However, this study used a fast-releasing H_2S donor, NaHS with a longer time of exposure (16 hours). Hence, future treatment with longer duration of slow-releasing H_2S donors might be a potential anti-inflammatory benefits for human.

Anti-inflammatory drugs are needed to reduce the progression of intravascular thrombosis, leucocytosis and sepsis (Gabay and Kushner, 1999). Anti-inflammatory treatment in atherosclerosis patients such as 3-hydroxyl-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors (statins) reduced the cholesterol biosynthesis (Kwak et al., 2003). This drug has effect on endothelial cells by decreased pro-inflammatory cytokines such as IL-8 and IL-6.

In recent findings, the CANTOS study showed the benefits of anti-IL-1β (canakinumab) as an anti-inflammation agent (Thompson and Nidorf, 2018). In patients with stable coronary disease and a raised hs-CRP level, canakinumab reduced primary and secondary outcomes (primary: nonfatal myocardial infarction, nonfatal stroke, cardiovascular death; secondary: primary plus urgent revascularization) (Ridker et al., 2017). Anti-IL-1β treatment has the potential to replace the conventional non-steroidal anti-inflammatory drugs (NSAIDs) which have adverse effects on cardiovascular outcomes (Salvo et al., 2011).

There are few anti-inflammasome drugs that are commercially available. Basically, the treatments are divided into two categories; targeting IL-1 and targeting inflammasome activation (Ahn et al., 2018). Other than canakinumab, anakinra is a commonly used drug as a recombinant IL-1 receptor antagonist, hence reducing IL-1β and IL-18 effects (Dinarello, 2011). The current drugs that

directly inhibit NLRP3 inflammasome activation are glibenclamide, MCC950, β-Hydroxybutyrate and cholesterol 25-hydroxylase (Lamkanfi et al., 2009). Interestingly, there is an increasing trend of studies involving the natural products, such as Aloe vera, red ginseng and curcumin, however these have not been commercialized as yet (Kim et al., 2018).

The anti-inflammatory role of H_2S remains controversial since both pro- and anti-inflammatory roles are widely reported (Castelblanco et al., 2017). Such contrasting effects might be mediated by different molecular mechanisms. Interactions with ion channel, protein *S*-sulfhydration or the modulation of cAMP varies the final outcomes (Wallace and Wang, 2015). However, the anti-inflammasome effects of H_2S are mediated by reduced xanthine oxidase activity, reduced mitochondrial ROS production, reduced ASC oligomerization and reduced caspase-1 activity (Castelblanco et al., 2017). Moreover, H_2S-based therapeutics are now being demonstrated in clinical trials, such as SG1002 for cardiovascular disorders and ATB-346 for arthritis (Wallace et al., 2018).

This thesis showed that both GYY4137 and AP39 exhibited anti-inflammasome properties. However, limited investigations were performed for confirmation of the inhibitory actions of H_2S donors. Even though this study revealed no obvious changes in morphology and amount of total protein in lysates, cell death might be compromising our interpretation of the inhibitory effect of H_2S (Guangdong et al., 2004). In the future, cell counts should be performed and measures such as trypan blue exclusion or measuring LDH release (Spencer et al., 2018).

Study of GYY4137 on LPS-treated human synoviocytes and articular chondrocytes showed reduction of pro-inflammatory cytokines. However, higher concentrations were used in this study (up to 500μM) (Li et al., 2013b). Moreover, the role of AP39 as an antioxidant was previously demonstrated in human kidney cells. AP39 has a possible role in the regulation of antioxidant enzyme, heme oxygenase-1 (HO-1) in mesangial cells and human podocytes, even though a higher concentration (10mM) was used in this study (D'Araio et al., 2014).

In conclusion, the precise role of H_2S in the inflammatory process is still uncertain. However, slow-releasing H_2S donors are more physiologically relevant and more relevant for development of potential drugs.

6.4 Signal 1 and Signal 2 NLRP3 inflammasome in endothelial cells

Our study involved HUVEC as a primary endothelial cell line and EA.hy926 cells as an immortal human endothelial cell line. There are two other commonly used immortal cell lines, HMEC-1 (human dermal microvascular endothelial cells) and ECV304 cells (spontaneous-transformed line from HUVEC) (Bouïs et al., 2001). However, HMEC-1 is more suitable for the studies involving lipoprotein and HDL effects (Muñoz-Vega et al., 2018).

There are a paucity of studies involving endothelial cells and inflammasome activation. Our results demonstrated HUVEC primed better with a combination of PMA and LPS as compared with LPS alone which can be explained by NF-κB activation due to PMA. Instead of LPS, further investigation of NLRP3 inflammasome priming in PMA stimulated and non-stimulated HUVEC might be investigated with mycophenolic acid. Mycophenolic acid reduced the activity of NF-κB and its inhibitor (IKBα) in priming the NLRP3 inflammasome (Huang et al., 2002).

We have not demonstrated any significant activation of Signal 2 inflammasome in HUVEC. It was well explained previously that vascular tissue and heart express lower levels of NLR and TLR inflammasome (Yin et al., 2009a). Furthermore, HUVEC have a limited life span and there is batch variation (Kurz et al., 2004). Further investigation of TNF-α and IFN-γ might be helpful in study of TLR and ASC upregulation as well as VCAM-1 expression in HUVEC (Shiohara et al., 2002).

Our results showed EA.hy926 cells exhibited Signal 1 expression, pro-IL-1β, following a combination of PMA and LPS treatment. This can be explained as PMA is a PKC activator in EA.hy926 hence triggering inflammasome activation. Furthermore, the effect of PMA also could be seen by the release of Signal 2 inflammasome end product, IL-1β in EA.hy926. It has previously been shown that

PMA upregulates hCAT-1-mediated L-arginine transport hence biosynthesis of the NLRP3 inflammasome (Gräf et al., 2001). Further study about ERK-dependant Egr-1 (early growth response protein 1) in EA.hy926 cells might be helpful. ERK induces TNF-α and activates NF-κB for NLRP3 inflammasome priming (Liu et al., 2016a). Moreover, LPS promotes MAPK and TLR4 activation to promote Signal 2 inflammasome activation (Xiang et al., 2015).

MicroRNA-233 is known for NLR signalling regulation (Haneklaus et al., 2012). Overexpression of microR-223 prevents accumulation of NLRP3 protein and inhibits IL-1β production. Further study of microRNA-233 in EA.hy926 cells might be beneficial to study the NLRP3 inflammasome regulation in promoting a pro-inflammatory phenotype in the endothelial cells (Li et al., 2014).

6.5 Limitations of this study

THP-1 cells are routinely used as a model for monocytes due to their uniform genetic background (Qin, 2012). Moreover, THP-1 cells and peripheral blood mononuclear cells (PBMC) demonstrated similar transcriptional pattern upon stimulation with LPS (Sharif et al., 2007). Even though most of the cytokines (IL-6, IL-8 and IL-10) released were comparable between THP-1 cells and PBMC, certain differences were observed upon stimulation with LPS. PBMC secreted higher amount of IL-1β and TNF-α over time and significantly increased after 24 hours LPS treatment (Schildberger et al., 2013).

Other than PMA, VD3 is one of the stimuli commonly used to induce macrophage differentiation in THP-1 cells (Daigneault et al., 2010). Both PMA and VD3 induce different signal pathways, which might result in different success in differentiation. VD3 mainly elevates the PKC-β expression, whereas PMA strongly increased PKC-δ expression (Schwende et al., 1996a). VD3 treated THP-1 cells have greater CD14 surface expression as compared to PMA-treated (Steinbach and Thiele, 1994). Hence, CD14 might binds LPS better, in the presence of lipopolysaccharide-binding protein (LBP). Furthermore, a previous study demonstrated, the resting period for 24 hours after the PMA exposure was the optimal protocol for the differentiation of THP-1 cells (Lund et al., 2016).

LPS acts as a priming agent of NLRP3 inflammasome (Hornung and Latz, 2010). Other than LPS, amyloid beta also might be useful in priming the NLRP3 inflammasome, and has been used mainly in microglial cells (Meda et al., 1995). Other than bzATP, nigericin has been used to activate Signal 2 NLRP3 inflammasome (Baroja-Mazo et al., 2014). bzATP-induced potassium efflux and maturation of IL-1β were equally matched by nigericin (Perregaux and Gabel, 1994). However, a longer duration was needed upon stimulation with nigericin which might cause apoptosis.

Our study demonstrated both endothelial cell types (HUVEC and EA.hy926 cells) did not show promising results in respect of Signal 2. EC have been termed "semi-professional" APC because they stimulate certain T cell immune responses *in vitro* (Rothermel et al., 2004). It was well documented that vascular tissues and heart express fewer types of TLRs and NLRs than immune and defence tissues (Yin et al., 2009a). The expressions of TLR and NLR in HUVEC were well-upregulated by TNF-α co-stimulation which might be beneficial for our future work. With that, other end point products of the NLRP3 inflammasome should be investigated such as HMGB1, pyroptosis and non-canonical cytokines, in endothelial cells. Expression of caspase-1 production in endothelial cells suggests pyroptosis cell death is reduced and would be helpful to study in future investigations (Bergsbaken et al., 2009).

H_2S is a toxic gas with reported pro-inflammatory properties (Basic et al., 2017). Limited investigations such as mitochondrial membrane potential (MMP), TLR4 and NF-κB obscured the molecular pathway of Signal 1 formation (Huang et al., 2016). Exogenous H_2S showed anti-inflammatory effects against FFA-induced inflammation and apoptosis in siRNA mediated silencing of TLR4 in monocytes (Luo et al., 2017). Administration of S-adenosyl-l-methionine (SAM), a CBS-specific agonist restored H_2S level and attenuated inflammation (Zhao et al., 2017b). Another slow-releasing H_2S donor (FW1256) showed NLRP3 inflammasome de-activation in primary mouse macrophages (Caleb, 2017). Furthermore, using CRISPR-mediated gene editing, absence of CSE decreased iNOS and COX-2 expression, supporting a role for H_2S as an anti-inflammatory agent.

Mice are the experimental tool of choice for the immunologist and the study of their immune responses mirrors the human immune system (Mestas and Hughes, 2004b). However, investigation of IFN-α promoting Th1 differentiation showed contradictory findings. The CANTOS study is a great example of anti-IL-1β treatment *in vivo* (Lembo, 2017).

Further *in vivo* study such as investigating the combination effect of intracellular superoxide generators with hydrogen sulfide donors might be of interest to demonstrate the clear role of redox modulation in the mitochondria on NLRP3 inflammasome activation.

In general, the investigations should be carried out in larger ‘n’ value to increase the statistical power. Further study in comparison with PBMC might be beneficial to delineate exact pathway in human NLRP3 inflammasome activation.

6.6 Conclusion

The first aim of this thesis was to investigate activation of Signal 1 and Signal 2 of the NLRP3 inflammasome in THP-1 cells and endothelial cells; HUVEC and EA.hy926 cells. Following differentiation of THP-1 with PMA, the cell was primed with LPS and activated with bzATP. As a macrophage model cell line, THP-1 cells exhibited obvious Signal 1 expression in the cell lysate and Signal 2 expression secreted in culture media. Even though both endothelial cell types showed Signal 1 expression, only EA.hy926 cells established the Signal 2 activation which was apparent upon combination treatment with PMA and LPS. Additionally, EA.hy926 exhibited HMGB-1 expression raising the possibility for other end products to be released extracellularly, and the subject of future study.

The second aspect of the thesis involved investigating mitochondrial dysfunction and NLRP3 inflammasome activation. Thus, the aim was to study the effects of intracellular superoxide generators; mitoPQ and PQ towards Signal 1 and Signal 2 NRLP3 inflammasome pathways. Only the higher concentration of mitoPQ was able to increase Signal 2 inflammasome in LPS-treated differentiated THP-1 cells. Furthermore, it was demonstrated that THP-1 was not primarily following the non-canonical pathway as pro-caspase-1 expression was observed in cell lysates and caspase 5 was not detected.

Lastly, we investigated other compounds which might antagonise mitochondrial function; GYY4137 and AP39 as slow-releasing H_2S donors. At the beginning, we found that 0.4% of DMSO was able to mask the effect of GYY4137 by significantly reducing Signal 1 and Signal 2 end products. Interestingly, higher concentrations of GYY4137 showed pro-inflammatory effects on Signal 1 activation. Even though higher concentrations of GYY4137 and AP39 did not replace the function of LPS, both compounds were able to act to inhibit the inflammasome as they reduced the Signal 2 NLRP3 inflammasome end products.

The results of this thesis are summarized as in Figure **6-1**:

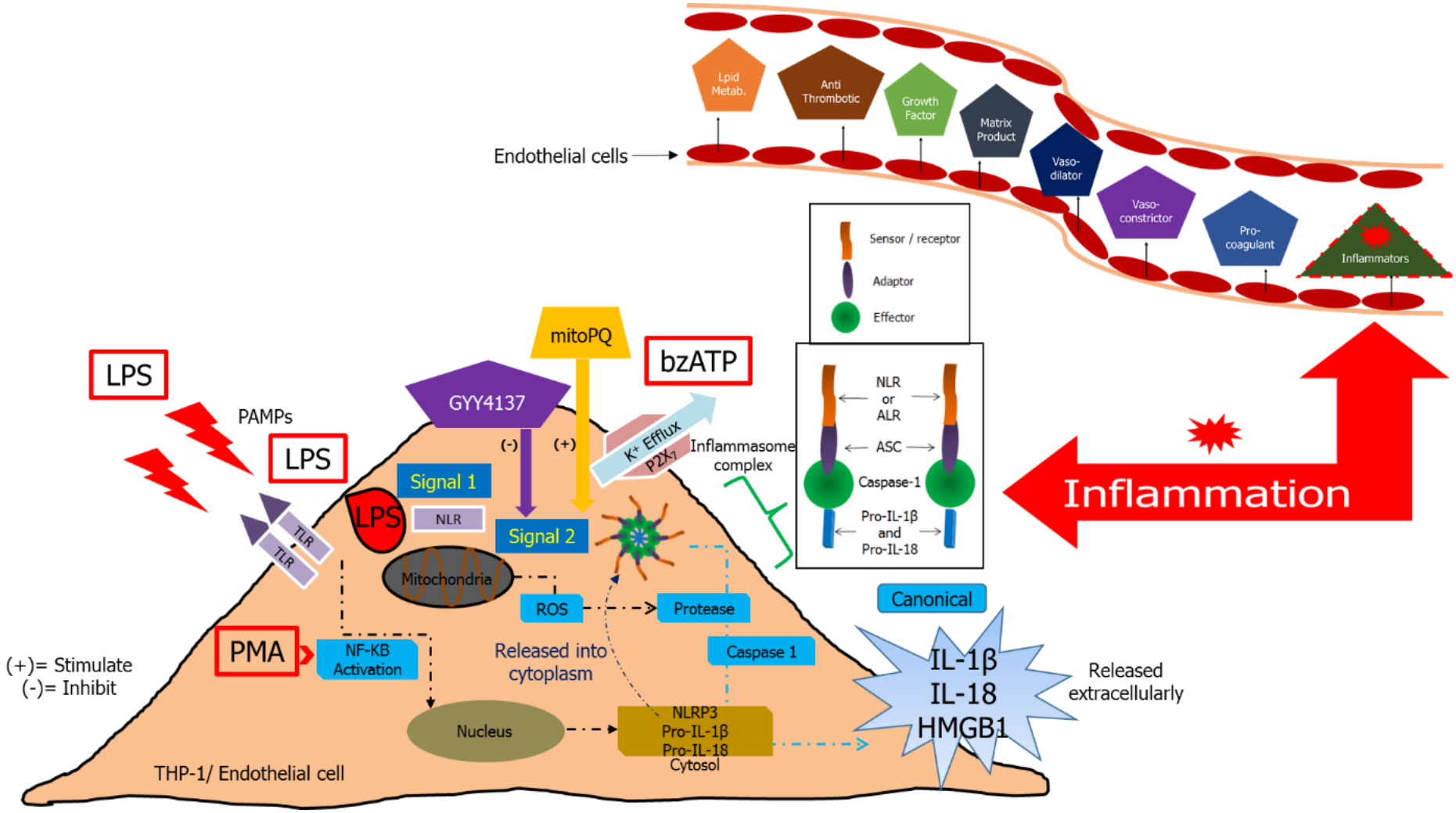
Endothelial cells
Lpid Metab.
Anti Thrombotic
Growth Factor
Matrix Product
Vaso-dilator
Vaso-constrictor
Pro-coagulant
Inflammators
Sensor / receptor
Adaptor
Effector
NLR or ALR
ASC
Caspase-1
Pro-IL-1β and Pro-IL-18
Inflammasome complex
Inflammation
LPS
PAMPs
LPS
TLR
TLR
LPS
mitoPQ
bzATP
GYY4137
(-)
(+)
K+ Efflux
P2X7
Signal 1
NLR
Signal 2
Mitochondria
ROS
Protease
Caspase 1
Canonical
PMA
NF-KB Activation
Released into cytoplasm
IL-1β
IL-18
HMGB1
Released extracellularly
Nucleus
NLRP3
Pro-IL-1β
Pro-IL-18
Cytosol
THP-1/ Endothelial cell
(+)= Stimulate
(-)= Inhibit

Figure 6-1 Schematic diagram showing Signal 1 and Signal 2 activation of NLRP3 inflammasome in THP-1 and endothelial cells

PMA was used to differentiate THP-1 cells to macrophages. Subsequent treatment with LPS primed the differentiated THP-1 cells. bzATP caused Signal 2 activation and release of end products (IL-1β and IL-18). There was no evidence of the non-canonical pathway in THP-1 cells. MitoPQ was able to increase Signal 2 expression in LPS-treated, differentiated THP-1 cells. Furthermore, H_2S donors were able to reduce Signal 2 NLRP3 inflammasome end products.

For endothelial cells, the cells were primed with the combination of LPS and PMA and showed Signal 1 expression. Only EA.hy926 cell exhibited Signal 2 NLRP3 inflammasome activation. EA.hy926 cells also demonstrated HMGB1 expression.

Appendix

Abstract

Abas R, Wozniak M, Herbert K (2016), *Emerging Role for the NLRP3 Inflammasome in Endothelial Cells* for British Society for Cardiovascular Research (BSCR) autumn meeting 2016, University of Leeds and for the festival of postgraduate research, University of Leicester 2017 (Poster).

Abas R, Wozniak M, Herbert K (2017), *NLRP3 Inflammasome Pathways in Endothelial Cells* for British Society for Cardiovascular Research (BSCR) autumn meeting, University of Oxford (Poster).

Abas R, Wozniak M, Herbert K (2018), *Mitochondria involvement in NLRP3 inflammasome pathways in monocytes and endothelial cells* for Frontiers of Cardiovascular Biology (FCVB), Vienna (Poster).

Abas R, Wozniak M, Herbert K (2018), *Modulation of NLRP3 inflammasome activation in human monocytes by mitochondria-targeted superoxide and hydrogen sulphide* for International Society for Heart Research (ISHR)- European section, Amsterdam (Poster).

Abas R, *Emerging role for the NLRP3 inflammasome activation in endothelial cells* for Malaysian Students Conference and Research Showcase (MySecon) 2017, University of Manchester (3-minute thesis).

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