The role of tyrosine phosphorylation in inflammasome complex formation and function

By

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For Dad, Mom, Joseph & Hannah

I. ABSTRACT

The inflammasome is a multi-protein intracellular complex formed following detection of immune ‘danger’ signals (pathogen-associated and damage-associated molecular patterns) that lead to the release of pro-inflammatory cytokines in the presence or absence of pyroptosis.

Protein phosphorylation, a reversible post-translational modification of proteins that results in the addition or removal of the phosphoryl group to a specific amino acid residue(s), is important in regulating cellular processes. Tyrosine phosphorylation has been shown to be essential in the regulation of the inflammasome and occurs at the level of the receptors (e.g., NLRP3, AIM2, and NLRC4) and adaptor protein, apoptosis-associated Speck-like protein with a CARD domain (ASC).

To identify the role of protein tyrosine phosphatases involved in inflammasome function, the protein tyrosine phosphatase inhibitor, phenylarsine oxide (PAO) was used to assess the activation of NLRP3, AIM2 and NLRC4 inflammasomes. We have found that PAO perturbs the formation of the inflammasome assessed by nucleation of ASC specks, and the processing and release of caspase-1 and IL-1β in both human and murine cells. Furthermore, we have demonstrated that PAO inhibits nigericin-induced global tyrosine dephosphorylation and ASC dephosphorylation implicating tyrosine dephosphorylation of the inflammasome and ASC as a necessary step in inflammasome activation.

Furthermore, by utilising site-directed mutagenesis, we identified putative tyrosine residues on ASC required for its function. The mutation of tyrosine residues to a non-phosphorylatable tyrosine mimic, phenylalanine, at residues Y60 and Y137

of ASC results in attenuated IL-1β release but not at Y36. Taken together, we have shown that PAO is a potent inhibitor of ASC dephosphorylation and consequently the NLRP3 and AIM2 inflammasomes suggesting an important role for tyrosine dephosphorylation in the activation of the inflammasome. In addition, we have demonstrated that phosphorylation of ASC at Y60 and Y137 is required for inflammasome function. However, more work is required to identify putative kinases/phosphatases involved in inflammasome regulation.

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III. PUBLICATIONS

Tyrosine dephosphorylation of ASC modulates the activation of the NLRP3 inflammasome

Bezaleel Mambwe, Kurt Neo, Hanif Javanmard Khameneh, Keith Weng Kit Leong, Maurizio Vacca, Richmond Muimo and Alessandra Mortellaro.

Submitted to Frontiers in Immunology (November 2018).

The inflammasome adaptor ASC intrinsically limits CD4^{+} T-cell proliferation to help maintain intestinal homeostasis

Hanif Javanmard Khameneh, Keith Weng Kit Leong, Andrea Mencarelli, Maurizio Vacca, Bezaleel Mambwe, Kurt Neo, Alicia Tay, Francesca Zolezzi, Bernett Lee and Alessandra Mortellaro.

Submitted to Frontiers in Immunology (February 2019).

IV. PRESENTATIONS

Poster Presentation: The role of tyrosine phosphorylation in inflammasome complex formation and function, **BCSR Autumn Meeting** (5-6 September 2018) Sheffield, UK

Poster Presentation: The role of phosphorylation in inflammasome complex formation and function, **The University of Sheffield Medical School Research Day** (14-15 June 2018), Sheffield, UK

Podium Presentation: The role of phosphorylation in inflammasome complex formation and function – 3-minute Presentation **SIgN Retreat** (March 2017), Indonesia/Singapore

Podium Presentation: The role of phosphorylation in inflammasome complex formation and function, **IICD DRIP Meeting** (4 May 2018), Sheffield, UK

V. ABBREVIATIONS

AIM2	Absent in melanoma 2
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BMDM	Bone marrow-derived macrophages
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
CARD	Caspase recruitment domain
cDNA	Complementary DNA
EDTA	Ethylenediaminetetraacetic acid
GSDMD	Gasdermin D
IKKα	Iκb kinase α
IL-18	Interleukin 18
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
JNK	C-Jun N-terminal Kinases
kb	Kilobases
kDa	Kilodalton
LPS	Lipopolysaccharide
MOI	Multiplexity of Infection
mRNA	Messenger ribonucleic acid
NLRC4	NACHT, LRR and CARD domains-containing protein 4
NLRP1	NACHT, LRR and PYD domains-containing protein 1

NLRP3	NACHT, LRR and PYD domains-containing protein 3
NOD-like	Nucleotide-binding oligomerization domain-like receptors
OA	Okadaic acid
OVN	Sodium orthovanadate
PAO	Phenylarsine oxide
PCR	Polymerase chain reaction
PKC	Protein kinase C
PKD	Protein kinase D
PKR	Protein kinase R
PMA	Phorbol 12-myristate-13 acetate
poly(dA:dT)	Poly(deoxyadenylic-deoxythymidylic)
PP2A	Protein phosphatase 2a
PRR	Pattern Recognition Receptor
PTP1B	Protein tyrosine phosphatase 1b
Pyk2	Protein tyrosine kinase 2
qPCR	Quantitative polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain Reaction
SDS	Sodium dodecyl sulfate
SHP1	Src homology region 2 domain-containing phosphatase-1
SHP2	Src homology region 2 domain-containing phosphatase-2
Syk	Spleen tyrosine kinase
THP1DMs	THP1-derived macrophages
TNF**α**	Tumour necrosis factor alpha

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1. Introduction

1.1 Introduction

The human body is subjected to a host of attacks such as invading microorganisms, allergens and tissue injury. Invading microorganisms (such as bacteria and viruses), allergens and tissue injury are known to result in disruption of homeostatic functions leading to various diseases and disorders. It is, therefore, imperative that the body has a way of clearing invasion by microorganisms or tissue injury to maintain homeostasis. Clearing of pathogens or repair of damaged tissue is carried out by the immune system, which is the host defence mechanism that comprises of various biological processes and structures. The immune system comprises of two layers of defence that are named the adaptive and innate immune system (Sattler, 2017, Medzhitov, 2007). The adaptive immune system responds specifically to the invading pathogen and mounts a complex immunological response involving the activation of various cell types (e.g. B and T lymphocytes). Furthermore, the adaptive immune system is responsible for conveying immunological memory, where the immune system 'remembers' antigens previously encountered which consequently enables the body to quickly respond to repeated invasion by the pathogen (Pancer and Cooper, 2006). On the other hand, the innate immune system is an immediate, non-specific defence mechanism against host invaders or tissue injury (Medzhitov, 2007). The innate immune system consists of physical barriers such as skin, chemicals in the blood and various immune cells which all function to prevent or eliminate invasion of pathogens and tissue damage. When microorganisms breach the skin or tissue damage occurs, the innate immune system recognises these as danger signals and provides a non-specific response by recognising molecular patterns associated with invading pathogens or tissue damage. Recognition of these molecular patterns precedes the activation of an innate immune system process

called inflammation. Inflammation is defined as a physiological response to invading pathogens or damage signals and a vital component of the innate immune system in responding to invading pathogens and/or tissue damage (Medzhitov, 2008). The ultimate goal of activating the inflammatory response is to defend against microbial invasion or mitigating tissue damage by mounting a host defence against infection or activating the tissue repair pathway, all leading to homeostatic state restoration (Ashley et al., 2012). There are two types of inflammation namely acute and chronic inflammation. Acute inflammation is transient and is resolved following clearance of the pathogenic/non-pathogenic invasion or cellular damage (Ashley et al., 2012, Medzhitov, 2008). On the other hand, chronic inflammation is the persistence of acute inflammation where the inflammatory response is not resolved resulting in prolonged activation of inflammation. Chronic inflammation is implicated in the pathology of a host of diseases and disorders such as neurodegenerative diseases, obesity, cancer and many more (Amor et al., 2010, Monteiro and Azevedo, 2010, Coussens and Werb, 2002). Therefore, understanding the mechanisms underlying inflammation activation is vital in the development of therapies that help to mitigate the inflammatory component of these disorders and thus slow progression of the disease.

1.1.1 Inflammation Inducers

Inflammation inducers are classified into exogenous and endogenous inducers of inflammation (Ashley et al., 2012, Okin and Medzhitov, 2012) .

Exogenous inducers of inflammation are non-host generated “danger” signals that include microbes such as bacteria and non-microbial inducers such as allergens. A subset of microbial inducers possess pathogen-associated molecular patterns (PAMPs) that are detected by dedicated sensor proteins known as pattern recognition receptors (PRRs) (Okin and Medzhitov, 2012). Pattern recognition receptors can be classified into two groups, namely membrane-bound and cytoplasmic pattern recognition receptors. Membrane-bound PRRs mainly consist of receptors that are tethered to the plasma membrane and recognise extracellular stimuli. This receptor class consists of toll-like receptors (e.g. TLR4 involved in bacterial lipopolysaccharide detection) and C-type lectin receptors (e.g. Dectin1) (Takeuchi and Akira, 2010).

Cytoplasmic PRRs, on the other hand, are non-membrane tethered receptors that recognise intracellular/cytoplasmic damage associated molecular patterns (DAMPs) or PAMPs. They are subdivided into the NOD-like receptors and the Rig-I-like receptors. NOD-like (nucleotide binding oligomerisation domain-like) receptors structurally consist of a C-terminal leucine rich domain, central NACHT domain and a variable N-terminal domain involved in protein-protein interactions. There are various NOD-like receptors that recognise a host of different DAMPs and PAMPs (discussed later). On the other hand, Rig-I-like (retinoic acid-inducible gene-I-like) receptors also known as RLRs are mainly involved in the recognition of intracellular viruses. These receptors possess a C terminal domain (CTD) involved in viral RNA binding, a central helicase core domain and an N-terminal caspase recruitment domain (CARD) (Takeuchi and Akira, 2010).

Virulence factors also serve as inducers of inflammation but are not directly detected by PRRs. Instead, the effects of the virulence activity are detected. For instance, the exotoxins produced by Gram-positive bacteria form pores in the cell membrane of the cell and lead to K+ efflux. This efflux consequently triggers the NLRP3 (NACHT- leucine-rich-repeat and pyrin domain containing protein) inflammasome leading to release of pro-inflammatory cytokines. Non-microbial inducers such as irritants and toxic chemicals similarly activate the inflammatory response as virulence factors by the detection of the activities elicited and not the inducer itself (Mogensen, 2009, Ashley et al., 2012) .

Endogenous stimuli of inflammation are signals produced in the event of tissue stress, damage or malfunction. These signals can include intracellular constituents released upon necrotic cell death (e.g. ATP, calcium and HMGB1) and tissue damage signals such as Hageman factor (factor XII) in vascular endothelial damage. Furthermore, there are other inducers of inflammation that are associated with chronic inflammation such as the formation of crystals like monosodium urate crystals or advanced glycation end products (AGEs) in gout and type 1/2 diabetes, respectively (Ashley et al., 2012) .

1.1.2 Inflammation Mediators

The inducers of inflammation, exogenous or endogenous, trigger the production of various inflammatory mediators. The inflammatory mediators in turn lead to the alteration of the activity and functionality of tissues and organs downstream of inflammation activation. The mediators are either produced by cells or derived from

plasma proteins. For instance, pro-inflammatory cytokines such as TNF, IL-1 and IL-6 are predominantly produced by tissue-resident macrophages and mast cells and function to lead to the recruitment and activation of leukocytes such as neutrophils, monocytes and macrophages to the site of damage or infection. This recruitment is required to result in the resolution of the infection. For example, macrophages can function primarily as a “clean-up” cell, engulfing pathogens or cellular debris through a process called phagocytosis. In addition, macrophages can also produce cytokines that aid in the recruitment of more immune cells to the site of damage or infection leading to resolution of the danger (Varol et al., 2015). However, persistent unregulated production of pro-inflammatory cytokines by various cell types and the recruitment of more immune cells to the site of infection/damage can result in a pathological inflammatory response.

1.2 Inflammation in Disease

The insights gained in understanding the inflammatory response has been to a large degree in aid of finding treatments to a host of disease/disorders. Chronic inflammation contributes to the pathology of neurodegenerative diseases, type 2 diabetes, autoimmune disorders and many more (Amor et al., 2010). Neurodegenerative disorders such as Alzheimer's and Parkinson's disease are characterised by activation of the inflammatory pathway in the central nervous system (CNS). In Alzheimer's disease, there is an increase in TLR2 and TLR4 expression on microglial cells and increase in pro-inflammatory cytokines around amyloid-beta (Aβ) plaques. These characteristics consequently result in progressive neurodegeneration via the recruitment of T- cells. Similarly, Parkinson's disease is characterised by the increased expression of PRRs TLR2, TLR5 and CD14 in the CNS, activation of Natural killer (NK) cells and microglia. The pro-inflammatory cytokine TNFα, produced by adipocytes (in high amounts in obesity) can modulate and attenuate carbohydrate metabolism in a local or systematic fashion (Amor et al., 2010). This has been shown to contribute to the progression of type 2 diabetes, insulin resistance and obesity (Cefalu, 2009).

Understanding the mechanisms underlying the activation of inflammation is incredibly vital in producing treatments for various disorders and diseases where inflammation contributes greatly to the pathology. Part of the inflammatory response is an intracellular multi-protein complex called the inflammasome that forms after detecting inflammation inducers intracellularly.

1.3 The inflammasome

The inflammasome (name derived from *inflammation* and '*soma*' meaning body) is a large cytosolic multi-protein complex. It is involved in the innate immune response to two groups of 'danger signals' namely, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) leading to the maturation and secretion of pro-inflammatory cytokines specifically IL-1β and IL-18.

The inflammasome comprises receptor proteins, an adaptor protein and an effector protein that together interact leading to maturation and secretion of IL-1β and IL-18. Host and pathogen-derived 'danger signals' are potent agonists for inflammasome activating receptors resulting in recruitment of an adaptor protein which further recruits the inactive form of the effector protein, pro-caspase-1. Pro-caspase-1 undergoes catalytic cleavage into the active form, which in turn results in the cleavage of pro-IL-1β and pro-IL-18 to the active forms. The active IL-1β and IL-18 are then secreted into the extracellular matrix (Laudisi et al., 2014, Hara et al., 2013b, Cullen et al., 2015) (Laudisi et al., 2014, Hara et al., 2013b, Cullen et al., 2015), usually accompanied by a type of cell death called pyroptosis.

The release of IL-1β following inflammasome activation is not fully understood but was initially suggested to be a consequence of pyroptotic cells (Miao et al., 2011). Necrosis has also been shown to be a mechanism responsible for cytokine release (Cullen et al., 2015). It has been proposed that the release of IL-1β is dependent on stimulus strength and the extracellular requirement of IL-1β (Lopez-

Castejon and Brough, 2011) which would also predict whether cells enter the pyroptotic pathway.

1.3.1 Inflammasome-mediated cell death

Apart from pro-inflammatory cytokine release, another consequence of inflammasome activation is pyroptosis which has been observed to be caused by an infection with pathogenic bacterial, viral and fungal species (Bergsbaken et al., 2009). Much like apoptosis, pyroptosis is a type of programmed cell death but is dependent on caspase-1(Figure 1-1), caspase-4/5 and caspase-11 in mice rather than on apoptotic caspases. Some caspase-1 dependent processes can occur without the activation of pyroptosis such as cytokine release. However, the activation of the pyroptotic pathway is inherently inflammatory (pro-inflammatory cytokine release) and would be determined by the cell type, and the type and magnitude of the stimulus (Fink and Cookson, 2005, Bergsbaken et al., 2009).

Activation of pyroptotic cell death has recently been attributed to inflammatory caspase mediated cleavage of the protein gasdermin D (Ramos-Junior and Morandini, 2017). Gasdermin D (GSDMD) is a substrate of inflammatory caspases-1, -4, -5 and -11 (Kayagaki et al., 2015, Shi et al., 2017) which is encoded by the *GSDMD* gene. The GSDMD protein consists of an N-terminal 31kDa domain (GSDMD-NT) and a 22kDa C-terminal domain (GSDMD-CT). The inflammatory caspases not only mediate the processing of the pro-inflammatory cytokines but also the processing of GSDMD by cleaving the GSDMD-CT and GSDMD-NT thus removing the auto-inhibition mediated by GSDMD-CT. GSDMD-NT, the active fragment, has been observed to mediate an increase in membrane permeability by

oligomerising and forming pores in the plasma membrane by binding to phosphatidylinositol phosphatase (PIP) and phosphatidylserine which in turn leads to cell swelling and lysis (Liu et al., 2016). GSDMD-NT can also form pores by binding to plasma membrane lipids in bacteria and therefore able to mediate bacterial death *in vitro*, implicating a direct bactericidal effect on intracellular bacteria. In mammalian cells, GSDMD-mediated cell swelling and lysis takes place as a means of preventing the replication of intracellular pathogens as well as the release of pro-inflammatory cytokines (Liu et al., 2016).

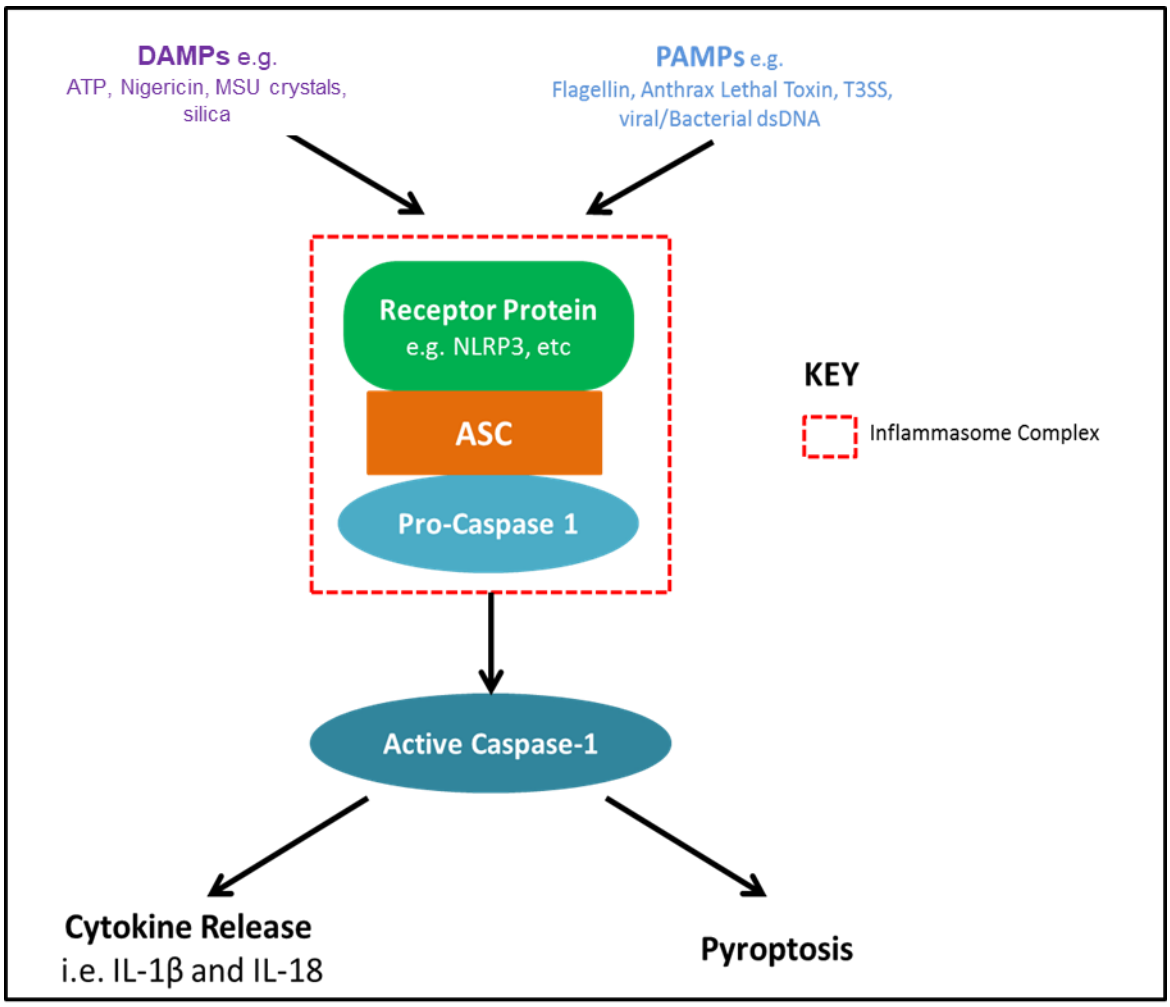

Figure 1-1: Inflammasome Activation Pathway
Activation of the inflammasome by various damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) leads to the cleavage of pro-caspase-1 into the active form which in turn results in the cleavage of pro-cytokines and the activation of pyroptosis via gasdermin D.

1.4 Types of Inflammasomes

There exist several different types of inflammasomes that undergo stimuli-dependent activation within the cell. Canonical inflammasomes are predominantly divided into two main subsets according to the receptor proteins that detect danger signals intracellularly. The majority of canonical inflammasomes currently studied fall within the nucleotide oligomerisation domain (NOD)-like receptor (NLR) subset and the non-NOD-like receptor subset, also called absent in melanoma 2. As such, NLRs are a specialized group of intracellular proteins that play a critical role in the regulation of the host innate immune response (Rathinam et al., 2012). Non-canonical inflammasome activation has also been identified where receptor and adaptor proteins are not involved in pro-inflammatory cytokine release. For instance, cytosolic LPS (e.g. from phagocytosed bacteria) is directly detected by caspase 4/5 (human) and caspase 11 (murine) leading to the recruitment of pro-IL-1β and its subsequent processing and release (Hagar et al., 2013, Viganò et al., 2015).

1.4.1 NLR inflammasomes

At least 34 genes encoding NLRs exist in the human genome, and several of these have been shown to form inflammasomes in response to various stimuli while the function of the others has not been demonstrated (Rathinam et al., 2012). NLRs are classed into two main subfamilies, which are the NLRs with a pyrin recruitment domain (NLRPs) and NLRs with a CARD recruitment domain (NLRCs). Generally, all NLRs consist of a common C- terminus leucine-rich repeat (LRR) domain and a central nucleotide-binding/oligomerisation (NACHT) domain. However, NLRPs possess an N-terminus pyrin domain (PYD domain) while NLRCs possess an N-

terminus caspase recruitment domain or CARD domain (Figure 1-2). During the activation of the inflammasome, the LRR domain seems to be implicated in ligand recognition. The NACHT domain is required for the oligomerisation of the activated receptor (Rathinam et al., 2012). Following detection of the ligand, the N-terminal domains, either PYD or CARD domains, function to allow the recruitment of the effector protein, pro-caspase-1 to enable it to be proteolytically cleaved.

The N-terminal domains as stated above are necessary to recruit pro-caspase-1. For instance, following injury or infection, the activated NLRP that possess the pyrin domain, such as NLRP1 or NLRP3, recruits the adaptor protein apoptosis-associated speck-like protein containing CARD domain (ASC) via a homotypic PYD-PYD interaction, to recruit pro-caspase-1. ASC is encoded by the *pycard* gene and consists of a pyrin and CARD domain. Its main function in the context of the inflammasome is to serve as an adaptor protein between the pyrin-containing NLRs and the CARD domain-possessing caspase. Thus, following detection of an inflammasome activator, the same NLRP receptors will oligomerise via the NACHT-NACHT domain interactions and followed by recruitment of ASC forming an ASC speck of oligomerised ASC protein. ASC oligomerisation serves as a platform for the recruitment of pro-caspases through their CARD domain (Figure 1-2) (Zambetti et al., 2012).

Initial studies reported that ASC was dispensable in NLRC signalling as observed in NLRC4 inflammasome formation and function. However, later it has been proposed that ASC is required to enhance the NLRC4 inflammasome activation in response to *Legionella pneumophila* (Case and Roy, 2011). These

results suggest that there is a stimuli/activator-dependant participation of ASC in the formation of a functional inflammasome in NLRC4 signalling where some NLRC4 activators also involve ASC for a full-blown activation.

In 2014, Si Ming Man *et al.* reported that ASC, in fact, forms a single ASC speck within the cell to which both NLRC4 and NLRP3 co-localise in a concentric filament-like structure with both NLRC4 and NLRP3 within a single circular ASC speck (Man et al., 2014). This is particularly interesting as it shows that the activation of different types of inflammasomes (in this case NLRP3 and NLRC4) does not proceed independently from one another but converges into the same ASC speck. Other NOD-like receptors have not been shown to interact in the 'single speck' and thus would be interesting to see whether the 'single speck' is a convergent point for all inflammasome related receptors.

In some experimental conditions, it has been noted that bacterial lipopolysaccharide (LPS) plays a priming role via the Toll-like receptor 4 (TLR4) and NF-κB pathway that triggers the expression of the inflammasome receptors and cytokine precursors. This has given rise to the two-signal or biphasic model of activation where the initial extracellular signal (Signal 1) by LPS involves the upregulation pro-inflammatory cytokines (pro-IL-1β and pro-IL-18). The second intracellular signal (Signal 2) by inflammasome stimuli, leads to the activation of the inflammasome and maturation of caspase-1 following the cellular internalisation of bacterial components (Bauernfeind et al., 2009, Baroja-Mazo et al., 2014).

The mechanism of action of inflammasome activation is poorly understood. The most extensively studied inflammasome is the one containing NLRP3 which has a range of activators (or stimuli) as shown in Table 1-1 (Baroja-Mazo et al., 2014). While various activators of this inflammasome exist, it remains unclear how structurally different stimuli can converge to elicit the activation of the NLRP3 inflammasome. It has been suggested the occurrence of a particular cellular event(s) is responsible for the subsequent activation of the inflammasome. In 2013, it was reported that most, if not all, of the NLRP3 inflammasome activators (nigericin, silica, Alum, MSU and ATP) initially lead to K^+ efflux and proposed this cellular event as possible convergent point triggering the activation of the NLRP3 inflammasome (Muñoz-Planillo et al., 2013).

In the case of the NLRC4 inflammasome, the activation step is preceded by the direct interaction of bacterial components (Type III secretion systems (T3SS) and flagellin) with co-receptor proteins, known as NOD-like receptor family apoptosis inhibitory proteins (NAIP). The NAIP receptors are a subfamily of the NLR proteins that possess an N-terminal LRR domain and three C-terminal Baculovirus Inhibitor of apoptosis protein Repeat (BIR) domains including a central NOD domain. It has been shown that some NAIPs directly bind to their ligand, for example, NAIP5 receptor binds to flagellin and T3SS components. Consequently NAIP5 associates to the NLRC4 receptor leading to the activation of the NLRC4 inflammasome (Gong and Shao, 2012).

1.4.2 AIM2 inflammasome

The receptor absent in melanoma 2 (AIM2), an interferon-inducible HIN-200 member, has been shown to recruit ASC and subsequently caspase-1 to form an inflammasome in response to cytosolic double-stranded DNA from viral and bacterial sources (Table 1-1). The AIM2 receptor consists of an N-terminal PYD domain and a C-terminal HIN-200 domain involved in DNA binding (Figure 1-2) (Hornung et al., 2009).

In experimental conditions, the presence of double-stranded DNA such as poly (dA:dT) in the cytosol results in the activation of the AIM2 receptor. This leads to the recruitment and formation of the ASC protein speck subsequently recruiting pro-caspase-1 via its CARD domain resulting in cytokine release (Hornung et al., 2009). Therefore, unlike NLRP3 and NLRC4, there is a direct interaction between the receptor and its ligand thus leading to activation of the AIM2 inflammasome. However, in contrast to the above mentioned NLRs, AIM2 has not yet been shown to be recruited within a single speck.

1.4.3 Pyrin inflammasome

The pyrin inflammasome consists of the receptor protein pyrin, ASC and caspase-1. Pyrin is encoded by the MEFV gene and is classified as part of the TRIM protein family that consists of a Bbox, coiled-coil and B30.2 domains (Heilig and Broz, 2018). Furthermore, it possesses the N-terminal pyrin (PYD) domain indicating that upon detection of a stimuli (such as bile analogues and bacterial toxins such as Clostridial TcdB) (Table1-1), the pyrin receptor can recruit the adaptor protein ASC

leading to caspase-1 processing and IL-1β release and pyroptotic death (Heilig and Broz, 2018).

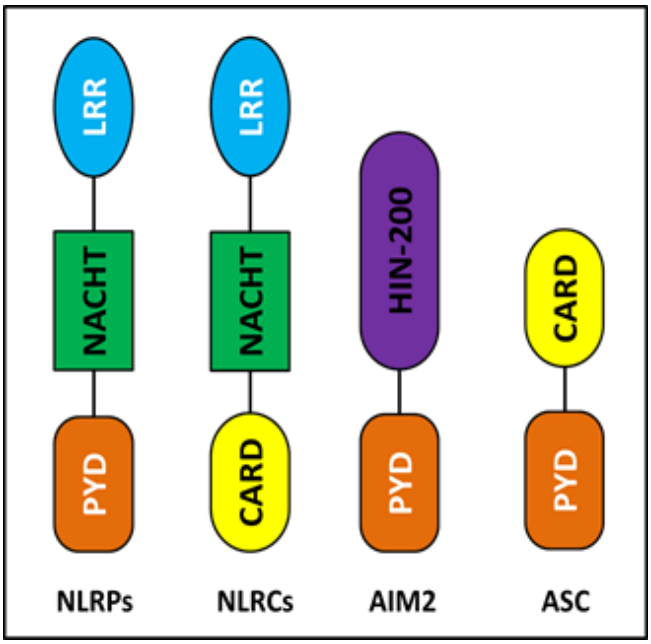

Figure 1-2: Schematic structures of the NOD-like receptors, AIM2 and ASC
NOD-like receptors possess a C- terminal leucine rich repeat (LRR) domain, a central nucleotide binding NACHT domain and either a PYRIN or CARD (caspase recruitment) domain at the N-terminal. On the other hand, the AIM2 receptor lacks the NACHT domain but possess a DNA-binding HIN-200 and a PYRIN domain. Apoptosis-associated speck-like protein with CARD (ASC) has two domains, a C-terminal CARD and an N-terminal PYRIN domain.

Table 1-1: Summary of elements involved in inflammasome activation

	COMPONENTS	**STIMULI**
NLRP1 inflammasome	NLRP1 ASC Pro-caspase 1	Anthrax lethal toxin
NLRP3 inflammasome	NLRP3 ASC Pro-caspase-1	Nigericin ATP Silica Alum Decrease in extracellular pH Monosodium urate (MSU) dsRNA Lysosomal damage Reactive Oxygen Species
NLRC4 inflammasome	NLRC4 Pro-caspase-1 ASC*	Bacterial Flagellin Bacterial Type III secretion systems (T3SS)
AIM2 inflammasome	AIM2 ASC Pro-Caspase-1	Bacterial and viral dsDNA
Pyrin inflammasome	Pyrin ASC Pro-Caspase-1	Bacterial toxins: *Clostridium difficile TcdB* *Histophilus somni IbpA* *Burkhoderia cenocepacia TecA*

**ASC is dispensable for NLRC4 inflammasome but some NLRC4 activators also involve ASC for a full-blown activation.*

1.5 The inflammasome and disease

Inflammatory involvement is well established in pathogenic as well as non-pathogenic disease processes. The production and activity of pro-inflammatory cytokines IL-1β and IL-18 have been shown to play vital roles in the progression of various diseases. Pro-inflammatory cytokines have been implicated in the progression of neurodegenerative diseases, for instance, Parkinson's disease (Yan et al., 2015, Codolo et al., 2013).

Mutations in the NLRP3 (also known as cryopyrin) have been identified to be the cause of rare autoimmune disorders collectively known as cryopyrinopathies. Chronic infantile neurologic cutaneous articular syndrome, familial cold autoinflammatory syndrome and Muckle-Wells Syndrome are examples of cryopyrinopathies that have been identified to be caused by mutations in the NLRP3 gene. There disorders are characterised by exacerbated pro-inflammatory cytokine levels and IL-1β targeted therapies have been the treatments of choice (Aksentijevich et al., 2007, Neven et al., 2008).

Inflammasome malfunction is also implicated in neurodegenerative diseases. Parkinson's disease is characterised by a loss of dopaminergic neurons in the substantia nigra of the brain. It is further characterised by the presence of Lewy bodies made up of fibrillar α-synuclein (α-Syn), a protein that has been shown to be involved in inflammasome activation and consequent pro-inflammatory cytokine production. α-Syn aggregates and activates the NLRP3 inflammasome in a phagolysosome disruption-dependent manner leading to the release of IL-1β similar

to crystalline and particulate matter, such as MSU, imject alum and silica (Codolo et al., 2013).

Furthermore, the loss of dopaminergic neurons also contributes to the activation of the NLRP3 inflammasome as dopamine negatively regulates the NLRP3 inflammasome via the dopamine D1 receptor (DRD1). Dopamine-induced activation of the DRD1 receptor leads to the production of cAMP, which binds to NLRP3 and promotes its ubiquitination by the E3 ubiquitin ligase, MARCH7 thus leading to NLRP3 degradation (Yan et al., 2015).

In addition, similar to Parkinson's disease, Alzheimer's disease characterised by amyloid-beta deposition was shown to have enhanced caspase-1 activation as a result of the protein aggregate-induced inflammasome (Tan et al., 2013).

Some cancers have inflammasome involvement embedded in some of the disease processes and progression (Kolb et al., 2014). Various inflammasomes have been shown to confer protection against tumorigenesis in colon cancers. For instance, the activation of the NLRP3 inflammasome leading to IL-18 production protects against colorectal cancer (Zaki et al., 2010) and similarly, the NLRP12 receptor negatively regulates colon tumorigenesis via the NF-κβ signalling pathway (Allen et al., 2012). Furthermore, also the NLRP6 and NLRC4 inflammasomes have been implicated in tumorigenesis and carcinogenesis (Chen et al., 2011, Normand et al., 2011, Hu et al., 2010). Inflammasome involvement in cancer is of particular importance therapeutically, that is targeting the inflammasomes can be carried out to elicit a protective effect and thus slow proliferation and progression of cancer.

Therefore, an in-depth understanding of the regulation of the inflammasome would be vital in developing the appropriate inflammasome-targeted cancer therapies.

The malfunction of the inflammasome is fast being established as a contributing factor in an array of diseases and disorder processes that include the above but also obesity and diabetes (Ting et al., 2006, Vandanmagsar et al., 2011, Dixit, 2013). Understanding the regulation of the inflammasome would be of great importance when considering treatments for any of the diseases discussed here not forgetting the others which may have implicated inflammasome function or malfunction intrinsic to the disease process such as fibrosis (Artlett, 2012).

1.6 Regulation of the Inflammasome

The dysregulation or lack thereof, of a cellular process results in gain or loss of function that often leads to disease and the inflammasome is not exempted. Regulation of the inflammasome can take place at various points within the inflammasome pathway; from activation, transduction of the activation signal and transcription to cytokine processing and secretion.

The regulation of the inflammasome can be carried out by two mechanisms; cell-extrinsic and intrinsic regulatory mechanisms.

1.6.1 Cell-extrinsic regulatory mechanisms

Cell-extrinsic mechanisms are regulatory mechanisms utilised by extracellular organisms such as viruses and bacteria to evade the inflammasome-mediated inflammatory response.

Viruses express molecules that possess anti-inflammasome properties. For instance, the Myxoma and Shope Fibroma virus express M013 and gp013L respectively which bind and inhibit the inflammasome adaptor protein ASC. Furthermore, viruses can also express proteins such as serpins that alter the proteolytic activity and thus inhibit proteolytic cleavage of inflammasome proteins such as pro-caspase-1 to its cleaved active form (Figure 1-3) (Rathinam et al., 2012).

Conversely, bacteria utilise various mechanisms to manipulate the activation of the inflammasome. *Yersinia pseudotuberculosis,* as an example, produces the protein YopK which binds to the translocon of the Type III Secretion System (T3SS). This binding masks the recognition site and thus inhibits the detection of the pathogen by the NLRP3 inflammasome (Zwack et al., 2015). Another strategy employed by bacteria is to differentially express proteins that can be detected by the inflammasome. *Salmonella* species utilise this strategy by down-regulating NLRC4-detectable flagellin and up-regulating the non-NLRC4 detectable SPI-1 T3SS which does not possess the motif required for detection by NLRC4 (Broz and Monack, 2011). Furthermore, enzymes such as phospholipases, cysteine protease, zinc metalloproteases and Rho GTPases are used by bacteria to alter the expression and/or activity of various components of the inflammasome (such as ASC and caspase-1) (Rathinam et al., 2012).

Cell-extrinsic mechanisms of regulation serve as a means of rendering the inflammasome ineffective against the micro-organism as observed in various bacteria and viruses thus preventing inflammasome activation and/or formation (Figure 1-3).

1.6.2 Cell-intrinsic regulatory mechanisms

Aside from the microbial-mediated regulatory mechanisms, cell-intrinsic mechanisms can control the activation and/or deactivation of the inflammasome. The cell-intrinsic mechanisms include the activation of signalling pathways as well as cellular processes. For instance, the activation of the AIM2 inflammasome in response to *Francisella tularensis* infection requires a functional Type I interferon (IFN) pathway

to boost IL-1β secretion. On the other hand, IFN signalling has been shown to inhibit NLRP3 inflammasome function via STAT1 leading to diminished IL-1β.

Other signalling pathways also modulate the inflammasome response and often include the use of second messengers, such as cAMP. Lee *et al.* (2012) reported that the activation of the mouse NLRP3 inflammasome is regulated by the murine calcium-sensing receptor (CASR) in a calcium ion (Ca^{2+}) and cAMP-dependent manner. The decrease in intracellular cAMP results in the activation of CASR which in turn leads to an increase of intracellular Ca^{2+} via phospholipase C and inositol-1, 4, 5-trisphosphate production and this increase in intracellular Ca^{2+} causes the assembly of the inflammasome. Furthermore, cAMP can negatively regulate the NLRP3 inflammasome by directly binding to NLRP3, and cAMP decrease removes this inhibition (Lee et al., 2012).

Another important process involved in regulation of the inflammasome is autophagy. Autophagy is a catabolic process involved in the degradation and renewal of intracellular components. The absence of the autophagic processing has been shown to augment the production of IL-1β and IL-18. Autophagy, therefore, is a negative regulator of the inflammasome (Rathinam et al., 2012).

Of particular interest are the biochemical regulatory mechanisms observed to take place in inflammasome function regulation. Ubiquitination is a post-translation modification of proteins that involves the conjugation of ubiquitin molecules to proteins that serves as a means of targeting a protein for transport, translocation or degradation (Mukhopadhyay and Riezman, 2007). Ubiquitination is carried out by

ubiquitin ligases in a ubiquitination cascade involving numerous enzymes, while deubiquitination is the removal of the ubiquitin molecules by enzymes called Deubiquitinating enzymes (DUBs) (Py et al., 2013). The NLRP3 receptor has been shown to be deubiquitinated in the NACHT and LRR domains following sensing of the stimuli of the NLRP3 inflammasome (Stutz et al., 2014). The murine protein BRCC3 protein (BRCC36 in the human) is a deubiquitinating enzyme that deubiquitinates NLRP3 upon activation leading to the assembly of the NLRP3 inflammasome (Py et al., 2013). Upon dopamine-induced activation of DRD1, cAMP can bind to NLRP3 and this leads to NLRP3 ubiquitination by the E3 ubiquitin ligase MARCH7 (Yan et al., 2015).

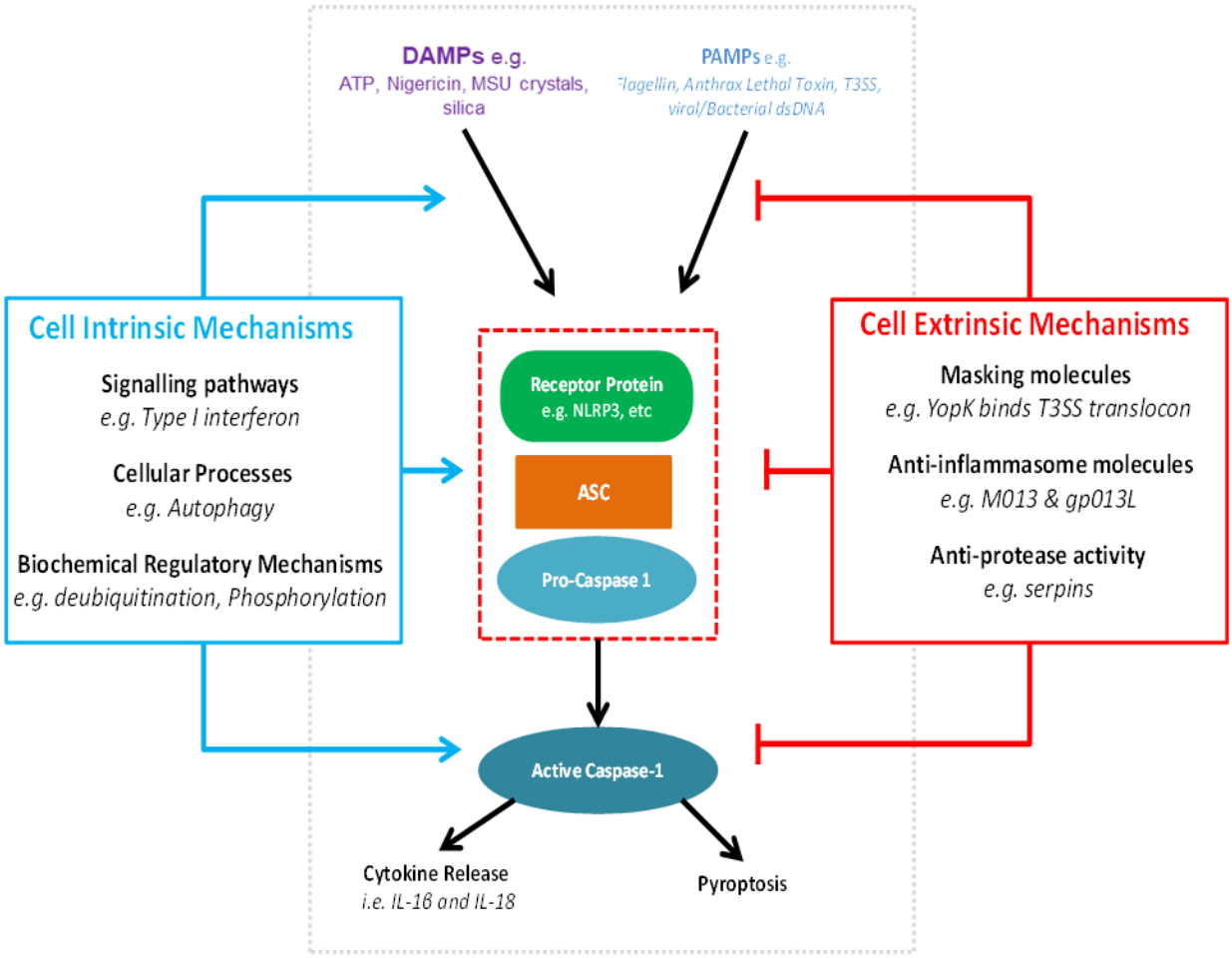

Figure 1-3: Summary of inflammasome regulatory mechanisms
Cell-intrinsic regulatory mechanisms ensure that activation of the inflammasome takes place normally while cell-extrinsic mechanisms elicited by microorganisms are utilised to evade the inflammasome.

1.7 Inflammasome regulation: the role of phosphorylation

Protein phosphorylation is a common post-translational modification that regulates most processes in the cell; it involves the addition of one or more phosphoryl groups to specific amino acid residues on a protein post-translationally. This can act as an activation or inhibitory modification of the target protein by causing conformational changes of the overall protein structure. Reversible phosphorylation is carried out by kinases and by phosphatases (Cohen, 2002). This vital cellular mechanism has been implicated in various pathways and cell processes.

*1.7.1 Amino acid phosphorylatio*n

Phosphorylation of proteins is a post-translational modification that takes place on various amino acid residues. The amino acids commonly modified in this manner include serine (S), threonine (T), tyrosine (Y) and histidine (H) (Cohen, 2002, Attwood et al., 2007).

1.8 Receptor phosphorylation

1.8.1 NLRC4 phosphorylation

The phosphorylation of NLRC4 at serine 533 (S533) takes place following cytosolic exposure to bacterial flagellin and type III secretion systems (T3SS).This leads to the formation of the NLRC4 inflammasome and maturation of caspase-1 (Qu et al., 2012). Matusiak *et al.* (2015) demonstrated that, upon infection with Salmonella enterica serovar typhimurium, S533 phosphorylation of NLRC4 follows a biphasic mechanism in which S533 phosphorylation initially serves as a priming step followed by the interaction of the flagellin carboxyl-terminus of NLRC4 with NAIP5 to form the inflammasome complex and lead to caspase-1 maturation, cytokine release and pyroptosis (Matusiak et al., 2015).

In another study, Qu Y *et al.* (2012) identified protein kinase C delta (PRKCD or PKCδ) as a candidate kinase for the serine 533 phosphorylation of NLRC4 in a kinase inhibitor screen. PRKCD is a ubiquitously expressed kinase involved in various cellular pathways, such as cell growth and cell death (Kikkawa et al., 2002). *Prkcd*$^{-/-}$ macrophages infected by *Salmonella typhimurium* (known to activate the NLRC4 inflammasome) showed a reduction of NLRC4 phosphorylation at S533 which resulted in the attenuated maturation of caspase-1 and IL-1β release (Qu et al., 2012).

The PKCδ-mediated phosphorylation of NLRC4 at serine 533 highlights a vital step in activation of the NLRC4 inflammasome following infection with bacteria. An indispensable step of receptor phosphorylation is seen to take place here. One can postulate that this post-translational modification, following cytosolic sensing of

inflammasome stimuli, may occur in other inflammasomes, such as the NLRP1, NLRP3, NLRP6 and AIM2.

1.8.2 NLRP3 phosphorylation

The NLRP3 protein activity is regulated by various kinases and phosphatases. It has been shown that NLRP3 can physically interact with the Bruton's tyrosine kinase (BTK), a cytoplasmic protein tyrosine kinase. The BTK-NLRP3 interaction was enhanced by nigericin treatment (signal 2) which led to inflammasome activation. Pharmacological and genetic inhibition of BTK activity resulted in attenuation of NLRP3 inflammasome function but not the AIM2 inflammasome suggesting NLRP3-specific regulation by BTK (Ito et al., 2015). Although the exact mechanism and target has not been elucidated, this result indicates that tyrosine kinase activity on NLRP3 is necessary for NLRP3 inflammasome activation and that BTK could serve as a potential therapeutic target, particularly in the reduction of ischaemic strokes induced by brain tissue injury.

Much like tyrosine phosphorylation, serine phosphorylation of NLRP3 by protein kinase D (PKD) results in NLRP3 inflammasome activation. Prior to activation, NLRP3 localises to the mitochondria-associated endoplasmic reticulum membranes (MAMs) but in response to inflammasome activators, the MAMs-NLRP3 complex seems to relocate to the Golgi apparatus. PKD is also recruited to the Golgi apparatus due to an increase in diacylglycerol (DAG) which consequently leads to phosphorylation of NLRP3 by PKD at serine 293 (S293). As a result, NLRP3 is released from the MAMs to form a functional inflammasome. The pharmacological

inhibition of PKD resulted in the suppression of the NLRP3 inflammasome activation (Zhang et al., 2017).

On the other hand, protein tyrosine phosphatase non-receptor 22 (PTPN22) has been shown to interact with NLRP3 upon inflammasome induction, which in turn dephosphorylates NLRP3 at tyrosine 861 (Y861). Loss of PTPN22 resulted in NLRP3 phosphorylation and little to no induction of mature IL-1β release. The presence of the PTPN22 619W is associated with an autoimmune phenotype characterized by overt IL-1β release similar to that observed in Crohn's disease, rheumatoid arthritis, type 1 diabetes and systemic lupus erythematosus (Spalinger et al., 2016).

Similarly, PP2A, a serine/threonine phosphatase, targets serine 5 (S5) in the pyrin domain of NLRP3. Okadaic acid-mediated inhibition of PP2A resulted in NLRP3 inflammasome inhibition (Stutz et al., 2017), revealing a dual role of PP2A in inflammasome activation by regulating NLRP3 activation and ASC localisation (see 1.6.3 ASC phosphorylation) (Martin et al., 2014).

In conclusion, both phosphorylation and dephosphorylation of the NLRP3 receptor protein are required for inflammasome formation and activation. However, the involvement of these opposing mechanisms appears contradictory, indicating that a complex system of regulation of the NLRP3 inflammasome exists where kinases and phosphatases simultaneously function to regulate the activation of the inflammasome.

1.8.3 ASC phosphorylation

Spleen Tyrosine kinase (Syk) and c-Jun N-terminal kinase (JNK) have been shown to take part in the regulation of the inflammasome by facilitating the formation of the ASC protein specks upon stimulation (Lin et al., 2015, Okada et al., 2014).

The murine adaptor protein, ASC, is phosphorylated at tyrosine 144 (Y144), a tyrosine residue residing in the CARD domain of ASC. In a kinase inhibitor screen, inhibitors of Syk and JNK led to the attenuation of the maturation of caspase-1 and production of IL-1β and IL-18. Furthermore, knockout of syk and *mapk8–mapk9* in peritoneal macrophages induced a decrease of IL-18 release and inhibited caspase-1 maturation following treatment with nigericin (NLRP3 activator) and dsDNA (poly(dA:dT), AIM2 activator), suggesting that the NLRP3 and AIM2 inflammasomes require Syk and JNK pathways for normal function. However, stimulation with *S. typhimurium* following Syk and JNK inhibition did not show a reduction in IL-18 production, suggesting that these kinases are dispensable for NLRC4 inflammasome-mediated IL-18 release. Syk and JNK knockout was sufficient to inhibit the formation of ASC specks, but the NLRP3-ASC interaction was unaffected indicating the requirement for ASC phosphorylation at Y144 in speck formation and caspase-1 activation (Hara et al., 2013a). Although Syk and JNK were shown to be necessary for ASC phosphorylation, they were not shown to physically interact with ASC. Consequently, pharmacological and knock down studies of the protein tyrosine kinase Pyk2 revealed the missing link between Syk/JNK and ASC. In 2016, Chung *et al.* found that Syk-mediated Pyk2 localisation to the ASC specks directly resulted in phosphorylation of ASC at Y144/6. (Chung et al., 2016).

While Pyk2-mediated phosphorylation of ASC via Syk positively regulates the activation of the NLRP3 inflammasome, Martin *et al.* (2014) reported that Iκβ kinase α (IKKα) activity is required to negatively regulate the NLRP3, NLRC4 and AIM2 inflammasome. In resting state macrophages, IKKα forms an intra-nuclear complex with ASC which functions to sequester ASC in the nucleus prior to activation. The phosphorylation of the serine residues S193 and S16 was shown to be vital for the IKKα-ASC interaction. In the biphasic activation mechanism of NLRP3 activation, the initial step of LPS priming resulted in the IKKα/ASC complex translocating into the perinuclear area facilitated by IKKi (IKK-related kinase). The second signal that is exposure to cytosolic ATP or nigericin, leads to NLRP3 inflammasome activation and, by inhibiting the activity of IKKα, the recruitment of PP2A to the perinuclear ASC/IKKα complex thereby causing the dissociation of ASC and IKKα. This allows ASC to interact with the other components of the inflammasome including caspase-1 (Martin et al., 2014).

Similar to PKCδ-induced NLRC4 phosphorylation, the activity of Syk and JNK kinases is required for normal ASC speck formation. However, whilst PKCδ was required to positively regulate the NLRC4 inflammasome, the IKKα activity negatively regulates the inflammasome and therefore revealed a dual role of kinases activity in regulating the inflammasome. Thus, a phosphorylation event could either be an activation or inhibitory modification of an inflammasome component.

The involvement of PP2A in inflammasome regulation has shown that phosphatases are also involved in inflammasome regulation and serve to allow inflammasome activation. Martin *et al*. (2014) showed that PP2A was vital in ASC

speck formation following activation of the NLRP3 inflammasome (Martin et al., 2014). It will be interesting to investigate further roles of PP2A other than the observed negative regulation of the IKKα-ASC interaction, as well as elucidate the role of other kinases and phosphatases involved.

1.9 Other kinases involved in inflammasome function

1.9.1 PKR role in inflammasome

Protein Kinase R is a double-stranded RNA (dsRNA)-dependent kinase that is activated by auto-phosphorylation when exposed to dsRNA (Yim and Williams, 2014).

Lu B. and colleagues (2012) had initially observed that HMGB1, a nuclear protein involved in DNA organisation and transcription, was released in response to poly (I:C) a PKR agonist and NLRP3 inflammasome activator (Lu et al., 2012, Rajan et al., 2010). In addition, they observed that PKR was phosphorylated and further determined that knockout and pharmacological inhibition of PKR perturbed the inflammasome response of caspase-1 maturation, and IL-1β and HMGB1 release. PKR knockout and inhibition with the PKR-specific inhibitors 2- aminopurine and $C_{13}H_8N_4OS$ (CNS) in LPS-primed and NLRP3-activated macrophages significantly disrupted caspase-1 maturation and cytokine release. Further, NLRP1, NLRP3, NLRC4 and AIM2 were shown to physically interact with PKR in a co-expression system, suggesting a regulatory role for PKR; however, no further information about the phosphorylation states of the receptors was reported (Lu et al., 2012).

In contrast, He *et al.* found that PKR was dispensable for NLRP3, NLRC4 and AIM2 inflammasome activation. They showed that inflammasome stimulation of macrophages obtained from two different strains of PKR knockout mice was not diminished compared to wild type. A clear explanation for the difference is not provided but they have suggested that differences in experimental conditions could

account for this differences and more work would be required to elucidate the role of PKR in inflammasome function (He et al., 2013).

Although the interaction between PKR and the various inflammasome receptors has been shown (Figure 1-4), there remains the question of the function and mechanism of this interaction. Yim and Williams (2014) proposed that the various receptor ligands could possibly interact with PKR first. Following the interaction, PKR would undergo auto-phosphorylation leading to PKR activation and interaction with the receptors. As consequence, the recruitment of ASC takes place (Yim and Williams, 2014). However, this model warrants further investigation.

1.9.2 The role of AMP-activated kinase

The AMP-activated protein kinase (AMPK) is a serine/threonine kinase activated by an increase in the intracellular AMP:ATP ratio in various conditions, such as hypoxia and exercise, and it possesses anti-inflammatory properties (Onyenwoke et al., 2012).

AMPK was shown to play a vital role in the monosodium urate (MSU)-mediated inflammatory (Figure 1-4) response in macrophages (Wang et al., 2014). MSU crystals inhibited AMPK phosphorylation at threonine 172 residue and thus led to an increased inflammasome response. Soluble urate is one of the hallmarks of gout which was able to give an insight into the inflammatory response as observed in this disease (Wang et al., 2014). In addition, the AMPKα1 knockout in bone marrow-derived macrophages (BMDMs) resulted in an enhanced inflammatory response to MSU crystals (Wang et al., 2014). Thus, AMPK phosphorylation, as for IKKα,

negatively regulates the activation of the inflammasome and that the loss and/or inhibition of AMPK showed exacerbation in the inflammatory response.

AMPK is known to interact with the phosphotransferase nucleoside diphosphate kinase (NDPK), a known histidine kinase (Onyenwoke et al., 2012, Attwood et al., 2007). AMPK inhibits the function of NDPK by phosphorylation of the serine 120 (Ser120) residue. Wang *et al.* (2014) reported that urate (either soluble or MSU crystals) inhibited the function of AMPK (Wang et al., 2014), suggesting that its inhibitory effect on NDPK could also be attenuated and thus NDPK would be active. As NDPK is a histidine kinase, this would warrant further study to establish the role, if present, of histidine phosphorylation in inflammasome activation and function.

Table 1-2: What is known in inflammasome component regulation by phosphorylation

Inflammasome component	What is known	Effect on inflammasome	References
NLRP1	Interacts with Protein Kinase R (PKR)	Unclear*	Lu et al2012, He et al 2013, Yim and Williams 2014
NLRP3	Interacts with PKR	Unclear*	
	Dephosphorylated by PTPN22 at Y861	NLRP3 Activation	(Spalinger et al., 2016)
	Interacts with BTK	NLRP3 Activation	(Ito et al., 2015)
	Ligand (MSU crystals) AMPK kinase inhibition	NLRP3 Activation	(Wang et al., 2014)
	Serine 5 dephosphorylation by PP2A	NLRP3 Activation	(Stutz et al., 2017)
	Interacts with PKR	Unclear*	(Yim and Williams, 2014, He et al., 2013)
NLRC4	Phosphorylated by PRKCD at serine 533	NLRC4 Activation	(Matusiak et al., 2015)
	Interacts with PKR	Unclear*	(Yim and Williams, 2014, He et al., 2013)
AIM2	Interacts with PKR	Unclear*	(Yim and Williams, 2014, He et al., 2013)
ASC	Phosphorylated at Y144/6 by Pyk2 in a Syk/JNK-dependent manner	Speck formation	(Chung et al., 2016)
	Interaction with IKKα requires serines 16 and 193	Inhibition by sequestration to intra-nuclear complex	(Martin et al., 2014)
	PP2A mediates IKKα –ASC dissociations	ASC free to interact with NLRP3	

**The data presently available regarding the role of PKR in inflammasome function is contradictory, where Lu et al (2012) showed PKR interacted with NLRP1, NLRP3, & NLRC4, and was indispensable for NLRP3 inflammasome activation and He at al (2013) showed it was dispensable for NLRP3, NLRC4 and AIM2 inflammasome's. (He et al., 2013, Yim and Williams, 2014, Lu et al., 2012)*

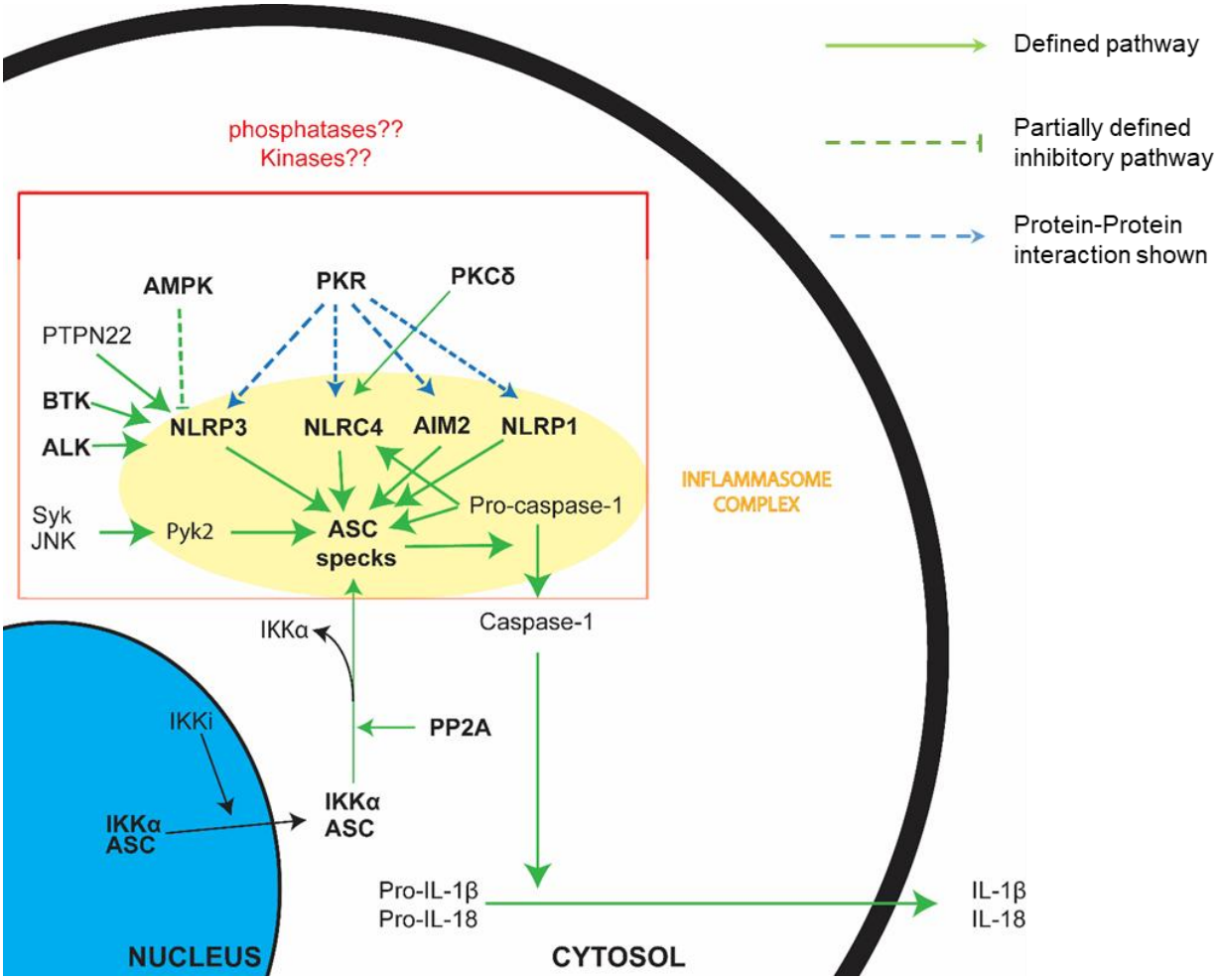

Figure 1-4: Kinases and phosphatases in inflammasome complex regulation

Although there are clearly defined pathways in the regulation of the inflammasome by phosphorylation, there still remain unknown kinases and phosphatases involved in the modulation of the inflammasome at various points of the inflammasome pathway such as at receptor activation, adaptor recruitment or proteolytic cleavage of pro-caspase 1 and pro-IL-1β.

1.10 Research Project

A number of studies have shown that kinase and phosphatase activities take place on inflammasome components thereby regulating the NLRP3 inflammasome. Although phosphorylation plays a pivotal role in inflammasome complex formation and function (Table 1-2), it is evident that there are substantial gaps in our current understanding. Several pathways involved in the phosphorylative regulation of the inflammasome are yet to be fully defined (Figure 1-4).

In order to address these gaps, some studies have elucidated kinase and phosphatase-mediated regulation of inflammasome activation. For instance, the NLRP3 protein has been shown to be dephosphorylated by PP2A upon signal 1 detection then phosphorylated by PKD upon signal 2 detection. Furthermore, NLRP3 has also been shown to interact with tyrosine kinase BTK on upon nigericin treatment and is also dephosphorylated by PTPN22, essential steps in NLRP3 inflammasome assembly (Spalinger et al., 2016, Stutz et al., 2017, Ito et al., 2015). This revealed that kinase and phosphatase activity can control inflammasome component activation. Therefore, it stands to reason that similar mechanisms of regulation could also take place on other inflammasome components such as ASC. ASC has already been shown to be a target for tyrosine phosphorylation but no tyrosine dephosphorylation has been shown. In this regard, we hypothesise that tyrosine dephosphorylation plays an important role in the regulation of the adaptor protein, ASC.

ASC is a convergent point for several receptors (NLRP3, AIM2, NLRC4) and effectors (caspase-1) and its absence results in complete abrogation of the downstream process (Kamo et al., 2013, Hanamsagar et al., 2011). Therefore, closing of the gaps in ASC phospho-regulation would aid in understanding inflammasome activation and thus lead to the development of treatments targeting inflammasome related diseases.

1.10.1 Project Aims

The main objective of this project is to provide a better understanding of the regulatory role of tyrosine phosphorylation of ASC in inflammasome complex formation and function.

As such, the aims of this project are:

- Determine the overall effect of tyrosine phosphatase inhibition on the NLRP3 inflammasome.
- Determine the impact of tyrosine phosphatase inhibition on ASC tyrosine phosphorylation, inflammasome formation and function
- Identify the putative tyrosine residues of ASC crucial for the formation and activation of the NLRP3 inflammasome.

2. Materials and Methods

2.1 Reagents

All the reagents used in this project are listed in Table 2-1.

Table 2-1: Materials and Reagents

Protocol	Name	Product Code	Company
Western Blot	IL-1β antibody	H-153	Santa Cruz Biotechnology
	ASC antibody	N-15	Santa Cruz Biotechnology
		N-15-R	Santa Cruz Biotechnology
		B-3	Santa Cruz Biotechnology
		AL177	Adipogen
		04-147 Clone 2EI-7	Millipore
	GAPDH antibody	MAB374	Millipore
	NLRP3 antibody	AG-20B-0014	Adipogen
	Anti-FLAG antibody	147935	Cell Signalling
	Caspase-1 antibody	C-20	Santa Cruz Biotechnology
		M-20	Cell signalling
	Goat X Rabbit HRP secondary	AP307P	Millipore

	Goat X Mouse HRP secondary	AP124P	Millipore
	Anti-GFP antibody	PA146326	Thermo Scientific
	Phosphotyrosine antibody (pY99)	sc-7020	Santa Cruz Biotechnology
	PP2A Y307 antibody	sc-12615	Santa Cruz Biotechnology
	Precision Plus Protein™ Dual Color Standards	1610374	Bio-rad
ELISA	IL-1β human ELISA capture antibody	14-7018-85	ebioscience
	IL-18 human ELISA capture antibody	D044-3	MBL
	TNFα human ELISA capture antibody	502802	Biolegend
	IL-6 human ELISA capture antibody	501102	Biolegend
	TNFα mouse ELISA capture antibody	14-7423-85	ebioscience
	IL-6 mouse ELISA capture antibody	504502	Biolegend
	IL-1β mouse ELISA capture antibody	840134	R&D

	IL-18 human ELISA detection antibody	D045-6	MBL
	IL-1β human ELISA detection antibody	13-7016-85	ebioscience
	IL-6 human ELISA detection antibody	501202	Biolegend
	TNFα human ELISA detection antibody	502904	Biolegend
	TNFα mouse ELISA detection antibody	13-7341-85	ebioscience
	IL-6 mouse ELISA detection antibody	504602	Biolegend
	IL-1β mouse ELISA detection antibody	840135	R&D
	Streptavidin-HRP	18-4100-51	ebioscience
	3,3',5,5'-Tetramethylbenzidine (TMB)	00-4201-56	ebioscience
Cell Culture & Treatment	Phenylarsine oxide	P3075	Sigma
	Sodium orthovanadate	S6508	Sigma
	RPMI-1640	11554516	Gibco
		BE12-702F	Lonza
		R8758	Sigma
	DMEM media	11965-084	Gibco

	Sodium Pyruvate	11360-070	Gibco
	Foetal bovine serum (FBS)	S181B-500	Biowest
	Phorbol 12-myristate 13-acetate (PMA)	P8139-10MG	Sigma
	IMDM media	12440046	Gibco
	Penicillin/Streptomycin	15140-122	Gibco
	Phosphate Buffered Saline (PBS)	SH30256.01	GE Healthcare
	Medium 199 media	M3769	Sigma
	Lipofectamine 2000	11668019	Invitrogen
	Opti-Mem media	31985-070	Gibco
	Lipopolysaccharide from E.coli O55:B5	ALX-581-013-L002	Enzo
	Nigericin sodium salt	J61349	Alfa Aesar
		N7143-10MG	Sigma
	Poly(dA:dT)	tlrl-patn-1	Invivogen
	Monosodium urate (MSU)	-	In house (SIgN)
Confocal Microscopy	Alexa Fluoro 488 (Goat X Rabbit)	A11034	Life Technologies
	Alexa Fluoro 488 (Goat X Mouse)	A11001	Life Technologies
	Alexa Fluoro 568 (Donkey X Mouse)	A10037	Invitrogen

	Alexa Fluoro 568 (Goat X Rabbit)	A11011	Life Technologies
	Alexa Fluoro 633 (Goat X Mouse)	A21050	Invitrogen
	Alexa Fluoro 555 (Goat X Rabbit)	A21428	Life Technologies
	4',6-Diamidino-2-Phenylindole (DAPI)	D1306	Life Technologies
Other reagents	Q5 site-directed mutagenesis kit	E0554S	New England Biolabs
	Protein G Sepharose	6511-5	Biovision
	Qiagen RNeasy kit	74104	Qiagen
	Cytox96® Non-radioactive Cytotoxicity assay	G1782/G1781	Promega
	TRI Reagent	T9424-100ML	Sigma
	RNA-to-cDNA Reverse Transcription Kit	4387406	Applied Biosystems
	SsoAdvanced™ Universal SYBR® Green Supermix	172-5270	Bio-Rad
	Dimethylsulfoxide (DMSO)		Sigma
	Bovine Serum Albumin (BSA)		Sigma

2.2 Cell culture

The cells used in this investigation are the murine bone marrow-derived macrophages (BMDMs), THP1 and HEK293 cell lines. THP1 cells are a monocytic cell line derived from a leukaemia patient (Tsuchiya et al., 1980). HEK293 cells are an immortalised human embryonic kidney cell line (Graham et al., 1977) commonly used in *in vitro* studies involving transfection of plasmids due to the ease with which they can be transfected.

2.2.1 Generation of BMDMs

Ethical permission for the use of experimental animals from the Institutional Animal Care and Use Committee (IACUC) was obtained in order to work with C57BL/6J mice as outlined by the National Advisory Committee on Laboratory Animal Research (NACLAR) guidelines in Singapore. The mice were euthanized by increasing carbon dioxide (CO_2) concentration. Both the tibias and femurs of each mouse were surgically excised, and the bone marrow was isolated by centrifugation at 5000 x g for 5 minutes. The isolated bone marrow was then cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 20% macrophage colony-stimulating factor (M-CSF) for 8 days to induce progenitor maturation towards the macrophage lineage. In experiments involving an ELISA, lactate dehydrogenase (LDH) or qPCR read-out, the BMDMs were cultured in a 96-well plate at 100,000 cells per well to normalise for the number of cells. For western blot experiments, 10cm dishes were used at a confluence of 80-100% to maximise lysate yields.

2.2.2 THP1 cell differentiation

THP1 monocytic cells were differentiated into macrophage-like cells with 100 nM phorbol 12-myristate13-acetate (PMA) in RPMI 1640 medium (RPMI-1640) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% sodium pyruvate and 1% L-glutamine cultured at 37 °C in 5% CO_2. The THP1 cells (at 80-90% confluence in suspension) were initially treated with PMA overnight (treatment phase) followed by a day in PMA-free media (resting phase). On the third day, THP1-derived macrophages (THP1DMs) were used for inflammasome-activating treatments. In experiments involving an ELISA, lactate dehydrogenase (LDH) assay or qPCR read-out, the THP1DMs were cultured in a 96-well plate at 100,000 cells per well, normalising by the number of cells. For western blot experiments, 10cm dishes were used at a confluence of 80-100% to maximise lysate yields.

2.2.3 HEK293 cells culture

HEK293 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal bovine serum, 1% sodium pyruvate, 1% penicillin/streptomycin and 1% L-Glutamine.

2.3 Inflammasome Activation

2.3.1 NLRP3 inflammasome activation

The NLRP3 inflammasome was activated by treatment with nigericin, monosodium urate (MSU) crystals and ouabain (Oua). Prior to nigericin, MSU or Oua treatment, THP1-derived macrophages (THP1DMs) or bone marrow-derived macrophages (BMDMs) were treated with *E. coli* LPS (1 µg/ml) for 2.5 – 3 hours. Nigericin (10 µM) or ouabain (125 µM) was added to the cells for up to 60 minutes while MSU crystals (200-400 µg/ml) was added for 6 hours before harvesting of cell-free supernatant and lysate for further analysis.

2.3.2 Absent in melanoma 2 (AIM2) inflammasome activation

The AIM2 inflammasome was activated by treatment with poly(dA:dT). Prior to poly(dA:dT) treatment, the differentiated THP1DMs or BMDMs were treated with *E. coli* LPS (1 µg/ml) for 2.5 – 3 hours. Poly(dA:dT) (1 µg/ml) was transfected (with 1 µl Lipofectamine 2000) into the cells for 6 hours before harvesting of cell-free supernatant and lysate for further analysis.

2.3.3 NLRC4 inflammasome activation

The NLRC4 inflammasome was activated by infection with *Salmonella enterica serovar* typhimurium (S. *typhimurium*) at multiplicity of infection 10 (MOI10). The THP1DMs and BMDMs were cultured in antibiotic-free medium followed by the addition of *S. typhimurium* for 1 hour. Gentamycin (100 µg/ml) was then added and

the cells were incubated overnight, and cell-free supernatant was harvested for ELISA analysis.

2.3.4 MSU-induced peritonitis mouse model

MSU-induced peritonitis in C57BL/6 mice was carried out in collaboration with Dr. Hanif Javanmard Khameneh (SIgN). Age-matched mice were intraperitoneally injected with 3.5 mg in-house made MSU crystals, after 5 hours peritoneal cells were harvested by peritoneal lavage. Phenylarsine oxide (1 mg/kg) was injected 2 hours before MSU crystal injection (N=1). The mice were euthanized by increasing carbon dioxide (CO_2) concentration. Permission for the use of experimental animals from the Institutional Animal Care and Use Committee (IACUC) was obtained in order to work with C57BL/6J mice as outlined by the National Advisory Committee on Laboratory Animal Research (NACLAR) guidelines in Singapore.

2.4 Cell lysis and protein quantification

The treated cells were harvested from culture plates by either scrapping, trypsinisation or direct addition of lysis buffer to the cells. Cells lysis was performed using radio-immunoprecipitation Assay (RIPA) buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-buffered saline pH 8.0 supplemented with protease inhibitors (Roche), 1 mM phenylmethanesulfonyl fluoride (PMSF) and phosphatase inhibitor (1 mM sodium orthovanadate) on ice for 10 minutes. Following lysis, the lysate was centrifuged on in benchtop centrifuge at 16, 000 x g for 10 minutes at 4°C to remove insoluble and nuclear matter. The supernatant was assayed with colorimetric assays,

bicinchoninic acid (BCA) assay or Bradford protein assay using bovine albumin serum (BSA) as standard to determine protein quantity.

2.5 Protein Immunoprecipitation

One milligram of lysate was initially pre-cleared with protein G sepharose beads for one hour at 4 °C. The precleared mix was centrifuged in benchtop centrifuge at maximum speed for 30 seconds at 4 °C and the supernatant was collected and the primary antibody for the target protein was added. The antibody-supernatant mix was incubated for 2 hours at 4 °C with agitation. After incubation, fresh protein G beads were added to the supernatant-antibody complex overnight at 4 °C. Following incubation, the mix was centrifuged and washed twice with complete lysis buffer (RIPA buffer) and then incubated with 30-60 µl of Laemmli buffer for 5 minutes at 100 °C prior to running on an SDS-PAGE.

2.6 Acetone protein precipitation

To assess IL-1β and caspase-1 secretion, nigericin stimulation was carried out in serum-free media (RPMI-1640). Equal amounts (2-3 ml) of the supernatant was collected from each condition and individually precipitated for 1 hour at -20 °C in 8-12 ml acetone. The precipitated proteins were resuspended in Laemmli buffer and processed for SDS-PAGE. The equal volume of condition serum-free media served as the loading control for the SDS-PAGE.

2.7 Disuccinimidyl suberate (DSS)-mediated cross-linking

Following cell treatment, cells were harvested by scraping in ice-cold ethylenediaminetetraacetic acid (EDTA; 0.5 M). Following centrifugation at ≈425 x g for 10 minutes at 4 °C, cells were re-suspended in 500 µl lysis buffer (20 mM HEPES pH 7.5, 150 mM potassium chloride (KCl) and 1% NP-40/IGEPAL CA-630 with protease and phosphatase inhibitors) and lysed by shearing through a 21G needle at least 10 times. Fifty microliters (50 µl) of the lysate were taken as the whole lysate loading control for the western blot. The remaining lysate was centrifuged at ≈2655 x g for 10 minutes at 4 °C and the supernatant was transferred into a fresh tube. The pellet was resuspended in 500 µl PBS and freshly prepared 2 mM DSS was added to both supernatant and resuspended pellet and incubated for a minimum of 30 minutes at room temperature with agitation/rotation. Following centrifugation of both incubations at ≈2655 x g for 10 minutes at 4 °C, the supernatants were discarded and the cross-linking reaction was quenched with 60 µl Laemmli buffer [60 mM Tris-HCl (pH 6.8), 2% lithium/sodium dodecyl sulfate, 10% glycerol, 100 mM dithiothreitol (DTT) and 0.01% bromophenol blue] then boiled at 95 °C for 10 minutes before loading onto an SDS-PAGE (Khare et al., 2016).

2.8 Western Blot Analysis

Lysates (30-50 µg) or precipitated/oligomerised proteins were incubated in Laemmlli buffer [60 mM Tris-HCl (pH 6.8), 2% lithium/sodium dodecyl sulfate, 10% glycerol,100 mM dithiothreitol (DTT) and 0.01% bromophenol blue] for 5-10 minutes at 95-100 °C. The now denatured protein samples were separated by SDS-PAGE

followed by protein transfer to PVDF (Millipore) or nitrocellulose membranes (Bradford). The membrane was blocked with 5% milk in TBS-Tween-20 at room temperature for 1 hour and then incubated overnight with primary antibodies (Table 2.1.1) as indicated. Following overnight incubation, the membranes were washed 6 times for 60 minutes (10-minute washes) and then incubated with the respective horseradish peroxidase (HRP)-conjugated secondary antibody (Dako, Millipore) for 1 hour at room temperature. Upon incubation, the membrane was washed 6 times for 60 minutes (10-minute washes) and then developed with Supersignal™ West Pico chemiluminescence detection (Pierce) solution and visualised by Bio-Rad chemiluminescence imager (Chemidoc) or on photographic film.

2.8.1 Western Blot Quantification by densitometry

Immunoprecipitation images obtained by the Bio-Rad chemiluminescence imager (Chemidoc) where imported into the imageJ software and band intensities were obtained as raw data. In order to quantify the target phosphorylation, the total protein (ASC) is first normalised by dividing all the total protein values with the highest total protein value. This is then followed by dividing the target tyrosine phosphorylation band value with its corresponding normalised total protein value. Then the control (untreated) values from the 3 experiments are averaged and each condition value is divided by the average of the untreated control yielding a fold change compared to the untreated control.

2.9 Generation of ASC mutant plasmids

ASC tyrosine to phenylalanine (Y>F) mutants of the pEF6-ASC-GFP plasmid (Figure 2-1), were created using the Q5 site-directed mutagenesis kit (New England Biolabs). The tyrosine codons TAC or TAT were substituted to phenylalanine codons TTC. The primer design was carried out using the NEBbasechanger (http://nebasechanger.neb.com/) (Table 2-2) as recommended by the manufacturer's instructions. Following primer design, the polymerase chain reaction (PCR) was carried out according to the manufacturer's instructions and following the conditions reported in Table 2-3.

Table 2-2: Primers for Y36, Y60, Y64, Y137 and Y146 site-directed mutagenesis of ASC

Selected Residues	Primers
Y36	Forward- GCGCGAGGGC**ttc**GGGCGCATCC Reverse- AGCGGCACCGACAGCAGC
Y60	Forward- GGTCAGCTTC**ttc**CTGGAGACCTA Reverse- AGCTTGTCGGTGAGGTCC
Y64	Forward- CCTGGAGACC**ttc**GGCGCCGAGC Reverse- TAGAAGCTGACCAGCTTGTCGGTG
Y137	Forward- GGATGCTCTG**ttc**GGGAAGGTCC Reverse- AGCAGCCACTCAACGTTTG
Y146	Forward- GGATGAGCAG**ttc**CAGGCAGTGC Reverse- GTCAGGACCTTCCCGTAC

Table 2-3: Mutagenesis PCR Reaction Setup

STEP	**TEMP**	**TIME**
Initial Denaturation	98 °C	30 seconds
25 cycles	98 °C 72 °C 72 °C	10 seconds 30 seconds 30 seconds/kb
Final Extension	72 °C	2 minutes
Hold	4–10 °C	

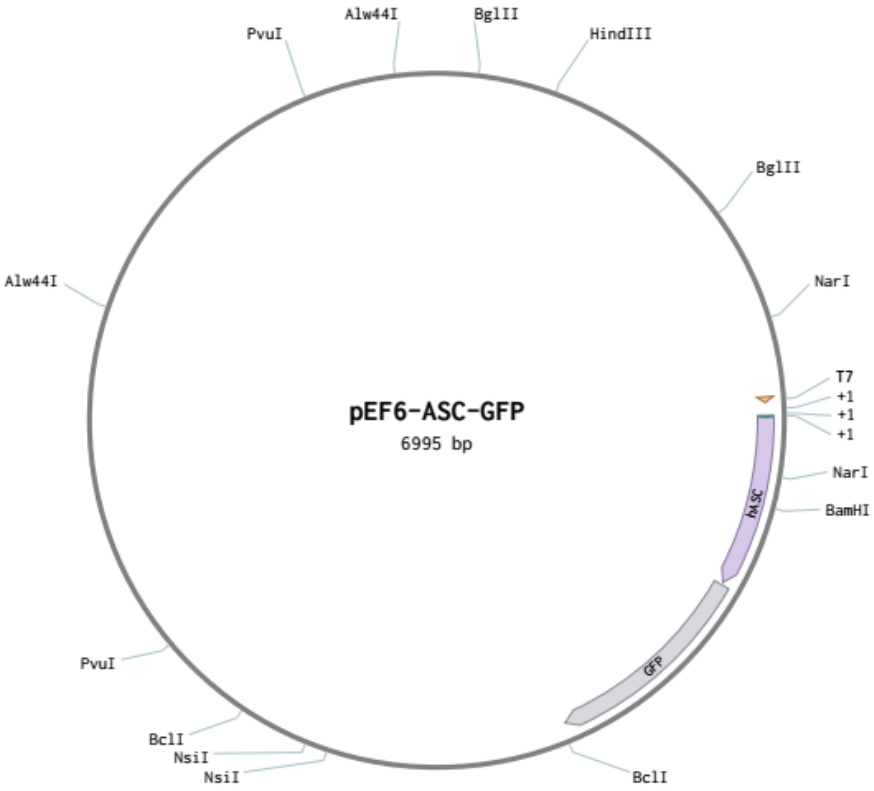

Figure 2-1: pEF6 ASC-GFP plasmid

The pEF6-ASC-GFP plasmid was kindly provided by Dr. Kate Schroder of the IMB Inflammasome Lab at the University of Queensland, Australia.

2.10 Bacterial transformation by heat shock

DH5α chemically competent *E. coli* were incubated with the plasmids encoding the inflammasome proteins on ice for 30 minutes followed by heat shock at 42 °C in a water bath for 45-60 seconds. The bacteria were then placed back on the ice for 5 minutes before the addition of SOC media and allowed to recover for 1 hour at 37 °C with agitation. After incubation, the bacteria were streaked onto agar plates containing carbenicillin (more stable alternative to ampicillin). Colonies were picked, grown in 5 ml cultures and a miniprep (Qiagen) was carried out according to manufacturer's instructions. The isolated plasmids were then sequenced to validate the site-directed mutagenesis.

2.11 Transfection of HEK293T cells

HEK293T cells were seeded into 24-well plates at a cell density of 200,000 cells per well, respectively and incubated overnight. The cells were then transfected with 100 ng pCR3-NLRP3-FLAG, 50 ng pEF6-ASC-GFP (and validated mutants), 30 ng pro-caspase-1, 100 ng pro-IL-1β and 720 ng empty vector (pCR3) with Lipofectamine 2000 for 24 hours. At 24 hours, the media was changed, and the cells were incubated for a further 24 hours. Forty-eight hours later, the media was collected for ELISA analysis and the lysate for western blot analysis.

2.12 Confocal microscopy

THP-1-derived macrophages cells were cultured in chamber microscope slides at a density of 150,000 per well/chamber. Following LPS priming, the cells were treated with nigericin for 30-45 minutes ±PAO. The cells were fixed with 1-2% paraformaldehyde at room temperature for 10-15 minutes. Following fixing, the cells underwent permeabilization (permeabilization buffer: 0.1% saponin, 0.2% gelatin, 5 mg/ml BSA and 0.02% sodium azide in PBS) followed by three 10-minute washes with PBS. Blocking solution (0.01% saponin, 0.2% gelatin and 5 mg/ml BSA in PBS) was then added for 45-60 minutes at room temperature, following which the primary antibody (anti-ASC, SCBT, N-15-R) (Table 2-1) was added for an hour at room temperature or overnight at 4 °C. The cells were washed 3 times for 10 minutes each with PBS then the secondary antibody conjugated to a fluorophore was added for 1 hour at room temperature in the dark. A final wash step was carried out with the addition of a DNA stain (4',6-Diamidino-2-Phenylindole (DAPI)) on the third wash. The images were taken with the Olympus confocal microscopy system (FV-1000 inverted Olympus IX81 microscope, magnification of 100X).

2.13 Cytokine Enzyme-linked Immunosorbent Assay (ELISA)

A 96-well plate was initially coated with the capture antibody (2 µg/ml) for the target cytokine (IL-1β and IL-18) for one hour at 37 °C or overnight at 4 °C. After incubation, the plate was washed 3 times with PBS-0.05% Tween-20 and dried, then blocked for 1 hour at room temperature with 1-2% BSA in PBS. The standard, a recombinant form of the cytokine of known concentration was added beginning at 1

ng/ml and serially diluted. Supernatant collected from treatment conditions was also added to the plate either as neat or up to a dilution factor of 10 and incubated for 2 hours at room temperature. The plate was then washed as before, and the biotin-conjugated detection antibody was then added for a further 1 hour 30 minutes at room temperature prior to washing again. Streptavidin-conjugated to horseradish peroxidase (HRP) was added and the plate was incubated at room temperature in the dark for 45-60 minutes. The plate was washed and following the wash step, 3,3′,5,5′-Tetramethylbenzidine (TMB), an HRP substrate, was added and the colorimetric reaction was stopped by 1 M H_2SO_4. The absorbance was detected at a wavelength of 450 nm using a plate reader.

2.14 RNA isolation

Ribonucleic acid (RNA) was isolated from cells using the Qiagen RNeasy kit (Qiagen). Following cell treatments, the supernatant was removed and TRIzol / TRI-reagent (to a maximum volume of 300 µl) was added to adherent cells and collected. Chloroform at 20% of the volume of TRIzol/TRI-reagent was added and the sample was centrifuged at 4 °C for 15 minutes (≈16,000 x g). The top supernatant (transparent) was immediately transferred into a fresh tube where 70% ethanol of equal volume was added. The sample-ethanol mix was then added to the RNAeasy mini column and centrifuged at ≈10621 x g for 60 seconds. Buffer RW1 (700 µl) was then added to the column and again centrifuged at ≈10621 x g for 1 minute. The column was washed with Buffer RPE twice before elution of the RNA with DEPC water at ≈10621 x g for 1 minute. RNA was then quantified by Nanodrop.

2.15 Reverse transcriptase-polymerase chain reaction

In order to obtain cDNA, Reverse Transcriptase- PCR (RT-PCR) was carried out on the isolated sample RNA with the High Capacity RNA–to-cDNA Reverse transcription kit (Applied Biosystems) under the conditions shown in Table 2-4.

Table 2-4: Reverse Transcriptase PCR Reaction setup

STEP	**TEMP**	**TIME**
Elongation	37 °C	60 minutes
Stop	95 °C	5 Minutes
Hold	4 °C	∞

2.16 Quantitative polymerase chain reaction

The quantitative PCR (qPCR) mix was prepared with a final forward and reverse primer concentration of 250 nM each and 100 ng of template cDNA sample for the target gene (IL-1β) and housekeeping gene GAPDH. The SYBR Green supermix (Bio-rad), a dye-based reaction mix was added (with nuclease free water) to a final volume of 20 µl. The PCR was then carried out on the ABI 7900HT real-time PCR system with the reaction steps shown in Table 2-5. The threshold cycle (Ct) values obtained were processed to obtain the fold change in gene expression by calculating the $2^{-\Delta\Delta Ct}$.

Table 2-5: Quantitative PCR reaction setup

STEP	TEMP	TIME
Stage 1	50 °C	2 minutes
Stage 2	95 °C	2-3 minutes
40 cycles	95 °C	15 seconds
	60 °C	1 minute

2.17 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) level in cell-free supernatant was assessed using the CytoTox 96® Non-Radioactive Cytotoxicity kit (Promega) according to manufacturer's instructions. The CytoTox® reagent (50 µl) was added to 50 µl of the samples and controls (whole lysate and media only) and incubated in a 96-well plate for 30 minutes in the dark. Then the stop solution containing acetic acid was added to stop the reaction. The absorbance was detected at a wavelength of 492 nm with a plate reader.

2.18 Flow cytometry

Peritoneal lavage exudate cells were incubated for 30 minutes at 4°C with the following antibody cocktail: anti-mouse CD11b PerCP-Cy5, anti-mouse, CD11c PE-Cy7, anti-mouse F4/80 Biotin, anti-mouse Ly6C FITC, anti-mouse Ly6G PE and anti-mouse MHCII APC-Cy7. This was followed by staining with streptavidin-APC for 30 minutes at 4°C and then DAPI was added to the samples 5 minutes prior to acquisition on FACS. The cells were washed and analysed on LSR II Flow cytometer (BD) with the following gating strategy: dendritic cells (live $CD11c^{+}$ $MHCII^{high}$), macrophages (live $CD11b^{+}F4/80^{+}$), neutrophils ($CD11b^{+}F4/80^{-}Ly6G^{+}Ly6C^{low/int}$), monocytes ($CD11b^{+}F4/80^{-}Ly6G^{-}Ly6C^{high}$).

2.19 Statistical analysis

Statistical analysis was carried out using the GraphPad Prism Software. All the data (with at least 3 experiments) was analysed by comparing the mean of each of the inhibitor treatment conditions to the LPS+Nig column using ordinary one-way ANOVA with the Dunnett post-hoc test without matching/pairing. Two-tailed paired t-test was carried out with Gaussian distribution assumed (parametric test) to analyse the number of ASC specks in confocal microscopy. Two-way ANOVA was carried out with Sidak post-hoc test. In the experiments involving varying concentrations of inhibitors and varying concentrations of vehicle, two-way ANOVA was carried out by comparing the inhibitor treatments and the vehicle treatments.

3. Effect of protein phosphatase inhibition on inflammasome function

3.1 Introduction

The inflammasome is a highly regulated complex within the cell. As already mentioned in this report, phosphorylation of inflammasome components is an indispensable regulatory mechanism that has been and is currently being studied extensively. Whilst much of the work has focused on kinase involvement (Lin et al., 2015, Chung et al., 2016, Okada et al., 2014, Hara et al., 2013b, Martin et al., 2014)**,** this study has sought to investigate the role of phosphatases in NLRP3 inflammasome function. In this chapter, the involvement of protein phosphatases is examined with the aid of phosphatase inhibitors to identify novel effects of phosphatase inhibition, thereby revealing new phosphatase-mediated NLRP3 inflammasome regulation mechanisms. The phosphatase inhibitors used are:

- Sodium orthovanadate (OVN), a broad-spectrum protein tyrosine phosphatase (PTP) inhibitor.
- Okadaic acid (OA), a selective inhibitor for the serine/threonine phosphatase, PP2A.
- PTP1B inhibitor (PTP1Bi).
- NSC-87877, a SHP1/2 dual inhibitor.
- Phenylarsine oxide (PAO), a broad spectrum PTP inhibitor (discussed in detail in Chapter 4)

3.2 Ethanol and DMSO inhibit the NLRP3 inflammasome

Most of the inhibitors used in this investigation could be dissolved in either ethanol, DMSO or water. It has already been reported that both ethanol and DMSO inhibit inflammasome activation (Ahn et al., 2014, Hoyt et al., 2016); therefore, prior to proceeding with our investigation, assessing and determining the effective concentrations of the vehicles would be vital.

THP1DMs were stimulated with *E. coli* LPS (signal 1) for 1 hour and then increasing concentrations of ethanol and DMSO for 30 minutes before the addition of nigericin to activate the NLRP3 inflammasome. Nigericin is an antibiotic that functions as a potassium ionophore leading to K^+ efflux, triggering the NLRP3 inflammasome (Muñoz-Planillo et al., 2013). To assess NLRP3 inflammasome activation, IL-1β ELISA was carried out. We found that both ethanol and DMSO vehicles inhibited the nigericin-induced NLRP3 inflammasome activation with ethanol exerting the most notable effect at higher concentrations when compared to DMSO (Figure 3-1). Therefore, we decided to use DMSO as a solvent for okadaic acid, PTP1Bi and PAO. Water was used for OVN and NSC-87877.

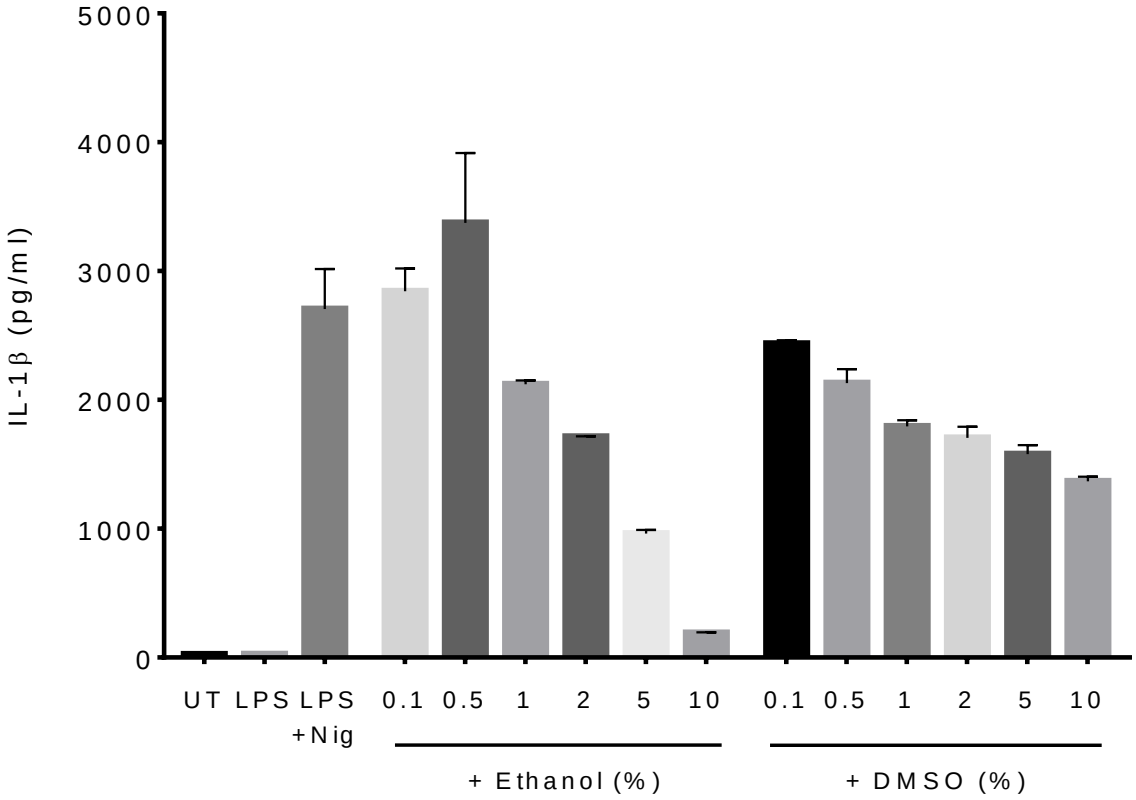

Figure 3-1: Ethanol and DMSO inhibit the NLRP3 inflammasome in THP1DMs

THP1-derived macrophages were primed with 1 µg/ml LPS for 3 hours followed by treatment with ethanol or DMSO (0.1% – 10%) for 30 minutes. Nigericin (10µM) was then added for 1 hour to activate the NLRP3 inflammasome. An ELISA of the culture media (supernatant) was carried out to measure IL-1β release (n=1)

3.3 Ouabain-induced NLRP3 inflammasome activation is inhibited by protein tyrosine phosphatase inhibitors

Ouabain (Oua) is a sodium-potassium (Na^+-K^+) ATPase inhibitor that causes an increase in intracellular sodium and depletion of intracellular K^+ which activates the NLRP3 inflammasome (Muñoz-Planillo et al., 2013, Kanneganti and Lamkanfi, 2013). In initial experiments, we carried out stimulation of primed THP1DMs with broad spectrum PTP inhibitors, including sodium orthovanadate (OVN) and phenylarsine oxide (PAO), for 30 minutes prior to the addition of 125 μM Oua for 1 hour.

We initially assessed the processing and release of IL-1β in the supernatant of treated cells and observed that IL-1β was cleaved and released in Oua treated cells, but this was completely abrogated in the presence of PAO (Figure 3-2A). We also observed that PAO treatment resulted in an increase in extracellular pro-IL-1β compared to Oua treatment, possibly associated with PAO-mediated decrease in processing and increased membrane permeability. Similarly, OVN treatment resulted in similar levels of extracellular pro-IL-1β suggesting a slight OVN-mediated inhibition of IL-1β processing but not complete abrogation since we observed the p17 IL-1β band in the supernatant blot. We further investigated the effect of PAO and OVN on global tyrosine phosphorylation. OVN had an expected inhibitory effect on global tyrosine dephosphorylation (Figure 3-2B), implicating some protein tyrosine phosphatases in mediating Oua-induced activation of the NLRP3 inflammasome. Compared to OVN, PAO did not exert as marked an inhibition on PTP activity in Oua-induced activation of the NLRP3 inflammasome as expected.

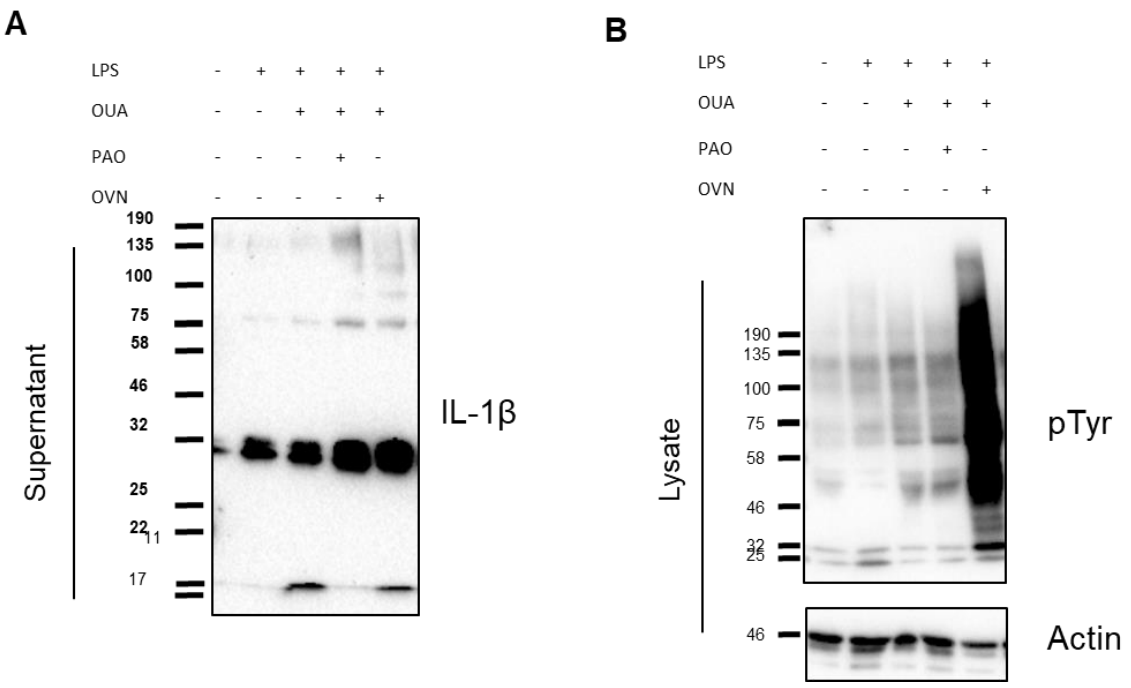

Figure 3-2: Ouabain-induced inflammasome activation is inhibited by PAO in THP1DMs

*THP1-derived macrophages were primed with 1 µg/ml LPS for 3 hours followed by treatment with broad-spectrum protein tyrosine phosphatase inhibitors, phenylarsine oxide (PAO (3 µM)) or Sodium orthovanadate (OVN (1 mM)) for 30 minutes. Ouabain (125 µM) was then added for 1 hour to activate the NLRP3 inflammasome. Immunoblot analysis of acetone precipitated supernatant or whole cell lysate was carried out to assess IL-1β processing and release (**A**) or tyrosine phosphorylation (**B**), respectively. Representative figures of n=3.*

3.4 Sodium orthovanadate treatment inhibits NLRP3 inflammasome function post-LPS-priming

Sodium orthovanadate (OVN) is a phosphate analogue that is commonly used as a broad spectrum protein tyrosine phosphatase inhibitor (Huyer et al., 1997) to investigate tyrosine phosphorylation. A previous study in THP1DMs showed that OVN treatment resulted in NLRP3 inflammasome activation evaluated as IL-1β release and pyroptosis (Ghonime et al., 2012). This study involved the treatment of THP1 cells with varying OVN concentrations over various durations ranging from 1 to 26 hours. In another study, Hoyt et al (2016) demonstrated that post-LPS OVN treatment (1 mM) further augmented ATP-induced IL-1β secretion in murine J774 cells (Hoyt et al., 2016), also suggesting that protein tyrosine phosphatase inhibition by OVN leads to inflammasome activation.

We investigated the effect of OVN on THP1DMs in nigericin-mediated NLRP3 inflammasome activation. We found that increasing OVN treatment significantly inhibited nigericin-induced NLRP3 inflammasome activation in LPS-primed THP1DMs as measured by IL-1β release by ELISA. Figure 3-3 shows reduced IL-1β secretion suggesting that, similarly to Oua-induced activation, nigericin-induced NLRP3 inflammasome activation is inhibited by OVN.

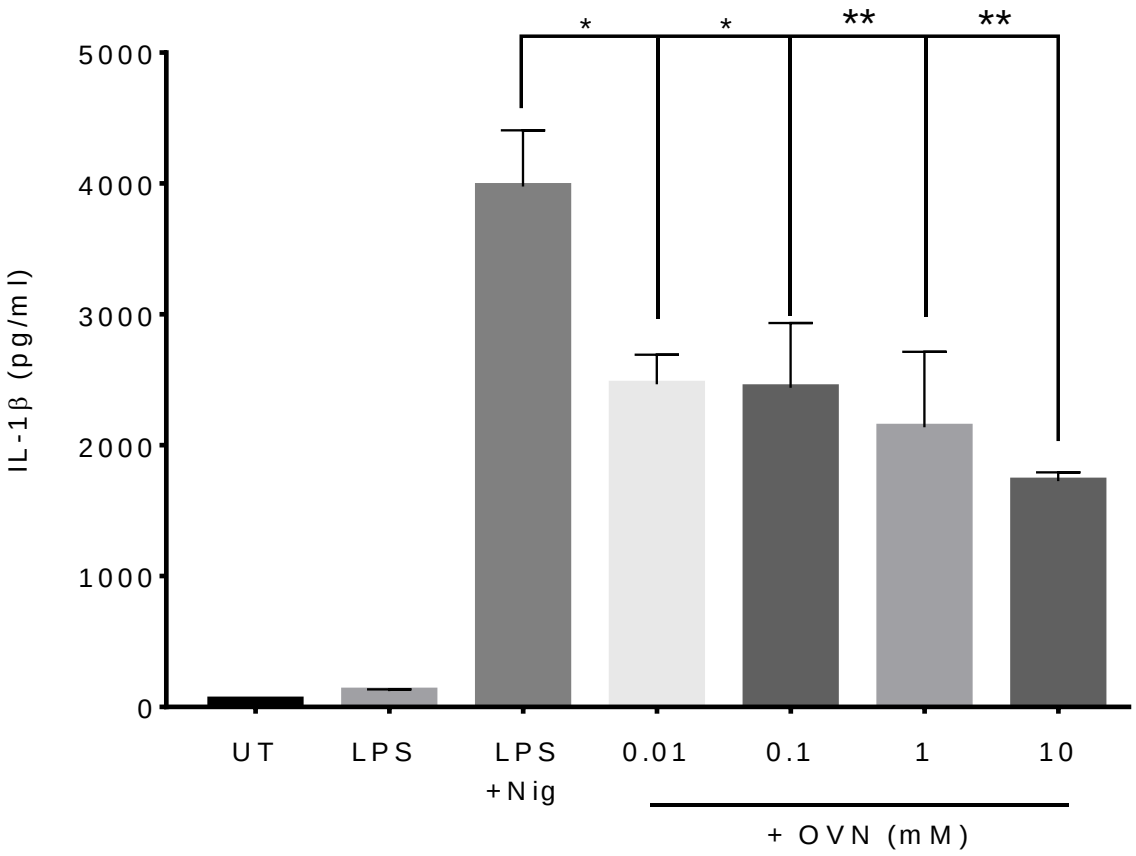

Figure 3-3: Sodium Orthovanadate treatment after LPS-priming inhibits NLRP3 inflammasome function in THP1DMs

*THP1-derived macrophages were primed with 1 µg/ml LPS for 3 hours followed by treatment with vehicle (water) or OVN (0.01 – 10 mM) for 30 minutes. Nigericin (10 µM) was then added for 1 hour to activate the NLRP3 inflammasome. An ELISA of the culture media (supernatant) was carried out to measure IL-1β release. All data represent the means ± standard error (SEM) Statistical analysis was carried out by comparing each of the inhibitor treatment conditions to the LPS+Nig column using Ordinary one-way ANOVA with the Dunnett test. Level of significance: *p≤0.05 **p≤0.01 (n=3).*

3.5 Okadaic acid treatment post-LPS stimulation has no effect on NLRP3 inflammasome function

Protein Phosphatase 2A (PP2A) is a serine/threonine phosphatase that has been shown to regulate inflammasome activation through direct serine dephosphorylation of NLRP3 (Stutz et al., 2017) and indirectly ASC cytoplasmic localisation via IKKα (Martin et al., 2014). Okadaic acid (OA) is a selective inhibitor of PP2A that has previously been used in murine inflammasome studies to establish PP2A as an indispensable phosphatase in inflammasome activation (Stutz et al., 2017, Martin et al., 2014). These previous studies demonstrated that okadaic acid-mediated inhibition of PP2A resulted in attenuated IL-1β processing and release.

The effect of PP2A inhibition by okadaic acid was explored in human (THP1DMs) cells in this study as opposed to previous murine studies. OA treatment was preceded by LPS stimulation then followed by nigericin treatment to assess the release of IL-1β as a measure of NLRP3 inflammasome activation. No significant effect on IL-1β secretion was observed following a 30-minute pre-nigericin treatment with varying concentrations of OA (Figure 3-4), suggesting no role for PP2A in human cells.

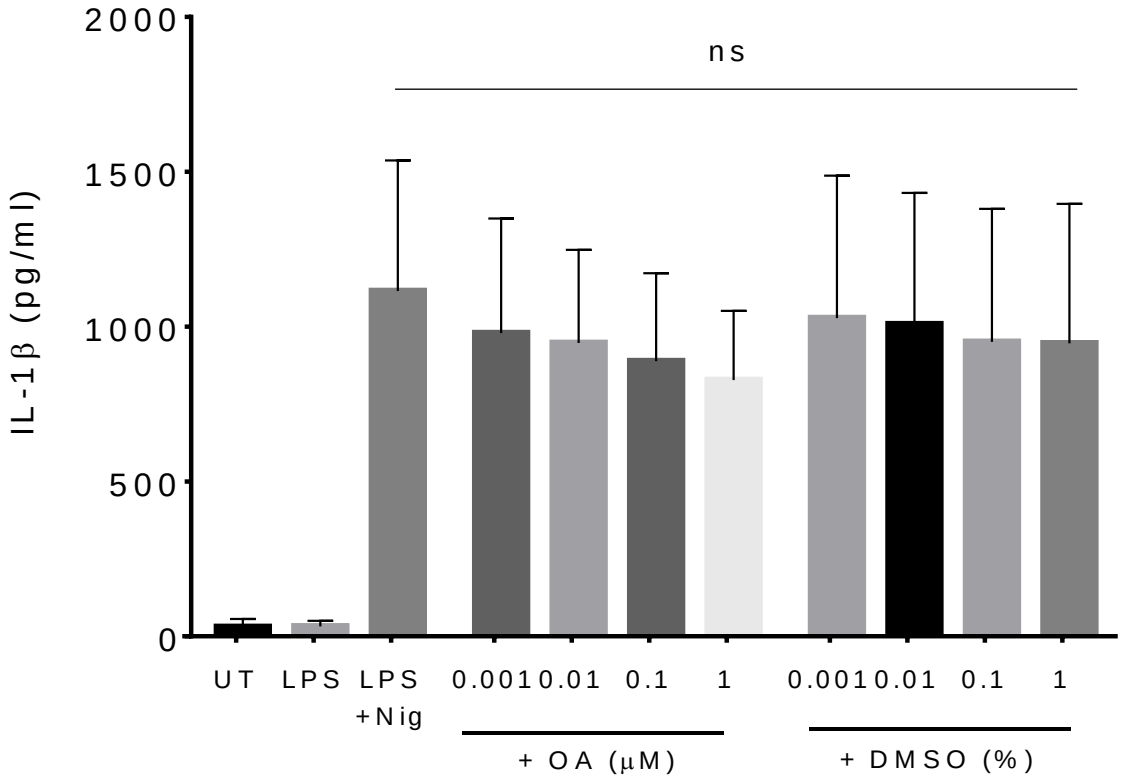

Figure 3-4: Okadaic acid has no effect on NLRP3 inflammasome function in THP1DMs

THP1-derived macrophages were primed with 1 μg/ml LPS for 3 hours followed by treatment with OA (0.001 – 1 μM) or with the corresponding vehicle concentration for 30 minutes. Nigericin (10 μM) was then added for 1 hour to activate the NLRP3 inflammasome. An ELISA of the culture media (supernatant) was carried out to measure IL-1β release. All data represent the means ± standard error (SEM) (n=3). Two-way ANOVA was carried out by comparing the inhibitor treatments and the vehicle (DMSO) treatments. Sidak post-hoc test was carried out. ns = not significant (p> 0.05)

3.6 Pharmacological PTP1B inhibition has no effect on NLRP3 inflammasome function

The serine/threonine phosphatase PP2A is regulated by a tyrosine phosphatase called **p**rotein **t**yrosine **p**hosphatase 1B (PTP1B) encoded by the *PTPN1* gene. PTP1B regulates PP2A activation by dephosphorylation of PP2A at tyrosine 307 (Y307) leading to its activation (Shimizu et al., 2003).

The investigation into PTP1B in the context of the inflammasome was to assess whether PTP1B plays a regulatory role in the inflammasome activation upstream of PP2A, as well as identify a novel function of PTP1B in the inflammasome complex. Therefore, 30-minutes incubation of THP1DM cells with a PTP1B pharmacological inhibitor (PTP1Bi) was carried out following 3 hours of LPS stimulation. PTP1Bi treatment resulted in attenuated function of the NLRP3 inflammasome, as measured by IL-1β release (Figure 3-5). However, despite increasing concentrations of PTP1Bi, we observed that IL-1β inhibition was not due to loss of PTP1B activity, but instead was a general effect exerted by the DMSO vehicle, which is known to inhibit NLRP3 inflammasome activation (Ahn et al., 2014). Indeed, the difference between the corresponding PTP1Bi and DMSO concentrations was not significant as analysed by Two-way ANOVA.

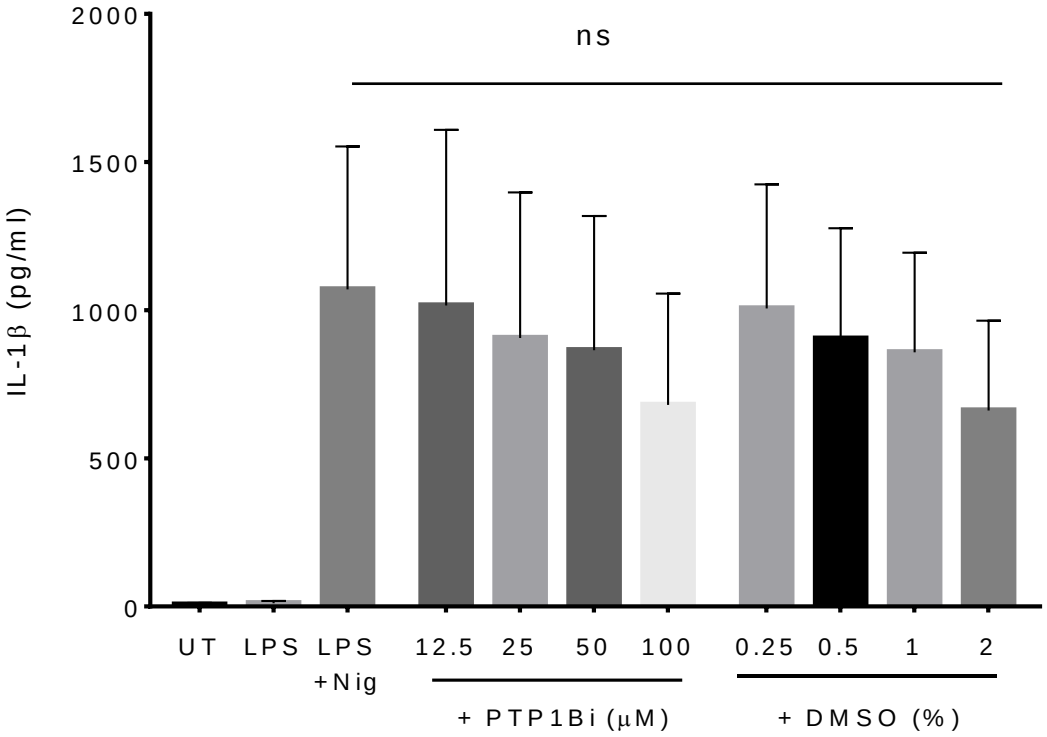

Figure 3-5: Pharmacological PTP1B inhibition has no effect on NLRP3 inflammasome function

THP1-derived macrophages were primed with 1 µg/ml LPS for 3 hours followed by treatment with PTP1B inhibitor (12.5 – 100 µM) or with corresponding vehicle (DMSO) concentration for 30 minutes. Nigericin (10 µM) was then added for 1 hour to activate the NLRP3 inflammasome. An ELISA of the culture media (supernatant) was carried out to measure IL-1β release. All data represent the means ± standard error (SEM) (n=3). Two-way ANOVA was carried out by comparing the inhibitor treatments and the vehicle (DMSO) treatments. Sidak post-hoc test was carried out. *ns = not significant (p>0.05).*

3.7 SHP1 and SHP2 are not involved in NLRP3 inflammasome function

Prediction software and online databases are valuable tools to quickly assess possible protein phosphorylation sites and protein interactions. The protein sequence of human *Pycard/ASC* was sourced from uniprot.com(Q9ULZ3) and used in the online consensus site database called ***PhosphoMotif Finder*** (http://hprd.org/PhosphoMotif_finder) (Amanchy et al., 2007). The protein tyrosine phosphatase SHP1 was predicted as a candidate tyrosine phosphatase for tyrosine residues Y36, Y64 and Y146 on human ASC as shown in Table 3-1.

Src homology region 2 (SH2) domain-containing phosphatase-1 (SHP1), also known as tyrosine-protein phosphatase non-receptor type 6, is a tyrosine phosphatase encoded by the *PTPN6* gene that is highly expressed in haematopoietic cell lineages (Plutzky et al., 1992). IL-1β release in murine neutrophils has been attributed to SHP1 negative regulation of the TLR and IL-1R signalling (Croker et al., 2011), but has not been shown in other cell types such as macrophages.

To assess whether SHP1 plays a role in NLRP3 inflammasome function in human macrophages (THP1DMs), pharmacological inhibition of SHP1 (and SHP2) was carried out using dual SHP1 and SHP2 inhibitor NSC-87877 (IC_{50}: 0.318 μM, 0.355 μM for SHP2 and SHP1, respectively). The NSC-87877 inhibitor (0 – 250 μM) did not affect IL-1β release induced by nigericin (Figure 3-6). Increasing NSC-87877 concentrations or incubation time of up to 4 hours (Figure 3-6A-C) did not result in any inhibitory or synergistic effect on IL-1β release. We observed that there was a

trend of increase in IL-1β release by increasing NSC-87877 concentration. Since we observed no effect at 1, 2 and 24 hours with increasing concentration, the experiment was not pursued further at that time.

Table 3-1: ASC possesses consensus sites for SHP1 at Y36, Y64 and Y146.
Human ASC amino acid sequence obtained from www.uniprot.com (Q9ULZ3 accessed:23/09/2017) was queried with the PhosphoMotif Finder (Amanchy et al., 2007) on the Human Protein Reference Database (http://www.hprd.org/PhosphoMotif_finder) for tyrosine kinase and phosphatase motifs.

Position in protein	Sequence in protein	Corresponding motif described in literature	Motif features described in the literature
36	EGY	[E/D]XpY	SHP1 phosphatase substrate motif
64	ETY	[E/D]XpY	SHP1 phosphatase substrate motif
146	EQY	[E/D]XpY	SHP1 phosphatase substrate motif

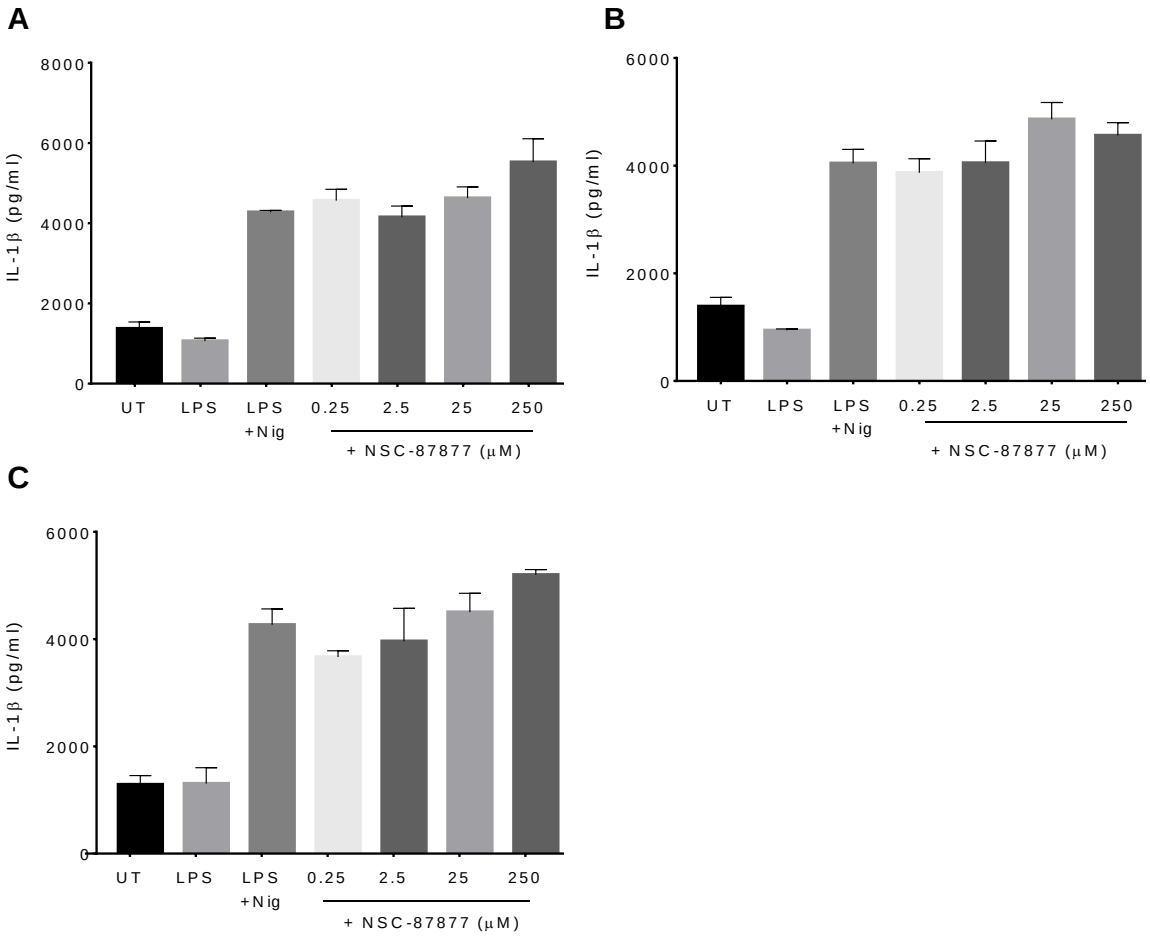

Figure 3-6: Pharmacological inhibition of SHP1 (and SHP2) has no effect on NLRP3 inflammasome function

THP1-derived macrophages were primed with 1μg/ml LPS for 3 hours followed by treatment with NSC-87877 (0.25 – 250μM) or vehicle for 60 ***(A)****, 120* ***(B)*** *and 240* ***(C)*** *minutes. Nigericin (10μM) was then added for 1 hour to activate the NLRP3 inflammasome. An ELISA of the culture media (supernatant) was carried out to measure IL-1β release (n=1).*

3.8 Discussion

The overall inhibition of tyrosine phosphatases by the broad-spectrum protein tyrosine inhibitors, PAO and sodium orthovanadate, resulted in a decrease in NLRP3 inflammasome-mediated IL-1β release, where PAO/OVN treatment was carried out after 3 hours of LPS stimulation for 30 minutes (Figure 3-1). This is in contrast to previous studies that have shown OVN-mediated protein tyrosine phosphatase inhibition resulting in enhanced IL-1β release (Ghonime et al., 2012, Hoyt et al., 2016). Ghonime *et al.* (2012) observed that OVN treatment of THP1DMs required at least 9 hours at a concentration of 50 μM (or 6 hours at 100 μM) to elicit significant cell death and IL-1β release. This effect was further enhanced by the addition of LPS overnight. Our data shows that varying concentrations of OVN treatment for 30 minutes sufficiently inhibited the inflammasome. On the other hand, Hoyt *et al.* (2016), showed that LPS-primed murine cells (J774 cells) treated with OVN at 1 mM for 1 hour prior to ATP-induced NLRP3 inflammasome stimulation released more IL-1β (Hoyt et al., 2016).

In this study, to target NLRP3 inflammasome activation directly and without affecting further gene transcription, inhibitor treatment was carried out 30 minutes prior to the addition of the NLRP3 inflammasome activator nigericin and ouabain and left on the cells together with the activator. As such, any effects on IL-1β release would be attributed to inhibition (or enhancement) of the NLRP3 inflammasome by the inhibition of direct protein-phosphatase interactions.

Differences in our experimental approach to that of Ghonime *et al.* and Hoyt *et al.* include cell species, OVN treatment duration and inflammasome agonist. Ghonime and colleagues produced THP1-derived macrophages (TDMs) with 200/500 nM PMA for 3 hours followed by 3 days of differentiation, whilst our THP1DMs were differentiated by overnight PMA stimulation, then an overnight resting phase. This difference in differentiation techniques could account for the difference in responses as proposed by Daigneault *et al.* (Daigneault et al., 2010) where different differentiation protocols resulted in differences in macrophage markers dependent on varying treatment protocols and thus differences in proteins expressed. Furthermore, following differentiation, the TDM cells were treated with low concentrations of OVN for long periods (up to 26 hours). This sustained OVN treatment could affect other pathways that in turn would lead to inflammasome activation, including further transcriptional activity. Reproducing the Ghonime *et al.* experiments in parallel with our experiments would be an interesting avenue to take as this would provide insights into how differentially differentiated THP1s (expressing a range of promonocytic to full macrophage markers) respond to inflammasome stimuli and how sustained OVN treatment affects cellular pathways.

In the Hoyt *et al.* (2016) report, various cell types were investigated for the effect of alcohol on inflammasome function, however only J774 cells, a murine macrophage cell line, were used to assess the effect of OVN on ATP induced NLRP3 inflammasome (Hoyt et al., 2016). Hagai *et al.* (2018) have reported that there is transcriptional variability across cell types and species. In that study bulk and single-cell transcriptomics in different cell types across various species were used to assess response to immune stimuli (Hagai et al., 2018). Therefore, although both

J774 and THP1DM cells similarly respond to NLRP3 stimuli, the cross-species transcriptome (and subsequently the proteome) may differ to allow OVN mediated IL-1β augmentation in ATP- stimulated murine cells and, conversely, inhibit nigericin induced IL-1β release in human cells.

NLRP3 inflammasome activation by ATP involves the activation of P2X7 receptors whereas nigericin functions as a potassium ionophore leading to K^+ efflux (Muñoz-Planillo et al., 2013). Further work is needed to elucidate the OVN sensitive pathways that are involved in ATP *vs.* nigericin induced inflammasome activation in both murine and human cells.

Our investigation into PP2A showed that there was no reduction in IL-1β release in okadaic acid-treated THP1DMs, whereas previous reports have suggested that PP2A is required for the activation of the NLRP3 inflammasome and thus inhibition of PP2A would inhibit IL-1β release (Stutz et al., 2017, Martin et al., 2014). In these reports, OA treatment was carried out prior to LPS stimulation in murine cells whereas OA treatment in our study was carried out post-LPS stimulation for 30 minutes in human cells. The difference in effect on IL-1β release to PP2A inhibition can be due to difference in treatment conditions (as is the case with OVN treatment) where the other reports would have had OA treatment lasting for the entirety of the experiment duration (up to 4.75 hours) (Martin et al., 2014) as opposed to only 1.5 hours in this investigation. Similar to OVN investigations, we used human cells and other reports used murine cells, which can also possibly account for the disparity in effect. This project's main focus was assessing and determining the role of PP2A in human macrophages (as opposed to murine cells as

done by previous studies (Martin et al., 2014, Stutz et al., 2017)). However, an interesting avenue to extend the scope of this study would be to carry out a comparative study with our conditions in murine cells. This approach would be crucial in furthering the understanding of the underlying mechanisms in PP2A mediated control of the inflammasome and identifying any differences (if any) in inflammasome control in murine versus human innate immune responses.

Since inhibition of PP2A yielded a different result to that previously observed in other reports, we hypothesized that okadaic acid was not sufficient to inhibit PP2A within the parameters of our experiment. Therefore, we sought to target the regulating tyrosine phosphatase of PP2A, PTP1B (Geraghty et al., 2013). PP2A activation requires dephosphorylation of tyrosine 307 (Y307) by PTP1B, therefore, targeting PTP1B was carried out to ultimately assess two main objectives: whether PTP1B is involved in regulating the NLRP3 inflammasome via PP2A and whether PTP1B directly regulates inflammasome complex proteins. Therefore, a PTP1B inhibitor was used to pharmacologically inhibit PTP1B in inflammasome–activated THP1DMs. As shown PTP1B inhibition with this inhibitor had no effect on the NLRP3 inflammasome. Nigericin-mediated inflammasome activation was not inhibited or enhanced by treatment with this inhibitor. This, therefore, showed that PTP1Bi treatment has no effect on inflammasome activation implicating no role for PTP1B. There are currently no reports on the role of PTP1B in inflammasome function, nor are there any reports implicating PTP1B to regulating PP2A during inflammasome activation, therefore, it would be of particular interest to further investigate this. This can be done by using alternative pharmacological inhibitors, protein knockdown with

siRNA or protein knockout then assessing PTP1B activity and if there is an effect on inflammasome function.

Phosphorylation of ASC on tyrosine residues is important for inflammasome function (Hara et al., 2013b, Chung et al., 2016). In a change of tack, rather than broadly inhibiting candidate protein tyrosine phosphatases by using broad spectrum inhibitors (PAO and OVN), we investigated the ASC protein for the presence of any consensus phosphorylation sites that have been previously identified in other proteins. Consequently, with the aid of the online Phospho-motif finder, we queried the ASC protein sequence to identify any tyrosine phosphorylation consensus sites. SHP1 was identified as a candidate tyrosine phosphatase for the ASC protein at tyrosines 36, 64 and 146. Therefore, we investigated whether SHP1 was required for NLRP3 inflammasome function. To achieve this, NSC-87877, a dual SHP1/2 inhibitor was used to treat THP1DMs after priming with LPS thereby focusing on processes taking place immediately before NLRP3 inflammasome activation. The SHP dual inhibitor had no effect on NLRP3 inflammasome function as shown by no decrease (or increase) in IL-1β secretion despite an increase in concentration or duration of post-LPS treatment. Although, ASC possesses consensus sites for SHP1, the data obtained shows that treatment with dual SHP1/2 inhibitor NSC-87877 in these conditions has no effect on the NLRP3 inflammasome. Croker *et al.* reported, however, that IL-1β release from murine neutrophils from SHP1$^{Y208N/Y208N}$ mice was enhanced (Croker et al., 2011). The *SHP1 Y208N* mutant is a substitution mutation of SHP1 where a tyrosine (Y) residue in the C-terminal SH2 domain is replaced with asparagine (N) rendering it inactive (Croker et al., 2008, Lukens and Kanneganti, 2014). This is a valuable tool in investigating NLRP3 inflammasome

function in other murine cell types of this mutant mouse such as macrophages, monocytes and dendritic cells. This would allow elucidation of the role of SHP1, if present, in inflammasome complex formation and function.

Taken together, we have shown that PAO and OVN inhibit IL-1β release, but the other phosphatase inhibitors investigated (PTP1Bi, OA and NSC-87877) here do not affect NLRP3 inflammasome activation by nigericin. However, there are limitations in our study that should be noted: Except for PAO and OVN, we did not show whether the inhibitors were indeed inhibiting PP2A, PTP1B and SHP1/2 by assessing target engagement of the inhibitors by assessing their substrates or post-translational modifications (such as Y307 dephosphorylation in PP2A). Positive controls of the phosphatase inhibitor treatments would provide further confirmation that our results are indeed in contrast to published data particularly with regard to the role of PP2A in IL-1β release as shown in murine cells (Martin et al., 2014, Stutz et al., 2017).

4. Phenylarsine oxide as an inhibitor of the inflammasome

4.1 Introduction

Overall tyrosine phosphatase inhibition mediated by sodium orthovanadate (OVN) appeared to inhibit the function of the NLRP3 inflammasome in our conditions. OVN was added to the THP1DM cells for 30 minutes after 3 hours of LPS priming then nigericin was added for an hour; thus the cells were exposed to OVN for a total of 1.5 hours as opposed to more prolonged periods in other studies. In parallel with the other inhibitor treatments, PAO, a dithiol compound, was identified to possess tyrosine phosphatase inhibiting activity. PAO exerts its inhibitory effect by binding to proximal cysteines/vicinal thiols within the active site of tyrosine phosphatases (Christina et al., 1992). In the only reported use in the inflammasome context, PAO has previously been used to inhibit ATP-mediated caspase-1 maturation and externalisation in human monocytes (Laliberte et al., 1999). Therefore, we sought to study the effect of PAO treatment on NLRP3, NLRC4 and AIM2 inflammasome function in THP1DM and BMDMs by measuring inflammasome activation indicators which include caspase-1 and IL-1β maturation and ASC speck formation. Further to this, we sought to elucidate the mechanism by which PAO could be acting in the NLRP3 inflammasome.

4.2 Post-LPS PAO treatment does not inhibit pro-IL-1β transcription in BMDMs and THP1DMs

As reported in Chapter 3, PAO potently inhibited the release of IL-1β induced by ouabain in THP1DMs. Estrov et al (1999) described that PAO had the ability to inhibit NF-κB signalling (Estrov et al., 1999). However, in our treatment conditions, PAO treatment would not have an effect on the NF-κB mediated IL-1β transcription as it was carried out after LPS priming (activates the NF-κB pathway via TLR4 signalling). Therefore, to demonstrate this, we carried out a quantitative PCR of pro-IL-1β *mRNA* in BMDMs and a western blot of the protein level in THP1DMs following signal 1 (LPS) and signal 2 (nigericin) in the presence of PAO 30 minutes prior to signal 2. There was no general decrease in pro-IL-1β *mRNA* in the presence of PAO (although there is variability in the relative amount of mRNA across the different PAO concentrations) (Figure 4-1A). In this initial experiment, we did not observe a drastic decrease in IL-1β gene transcription in the presence of PAO. With this observation and the constraints of time, we were not able to repeat this experiment.

Figure 4-1B shows that THP1DMs expressed the same amount of pro-IL-1β in untreated and LPS only but this was diminished in the presence of nigericin as a result of inflammasome induced processing. PAO restored pro-IL-1β levels implicating inhibition of pro-IL-1β processing and not transcription and translation.

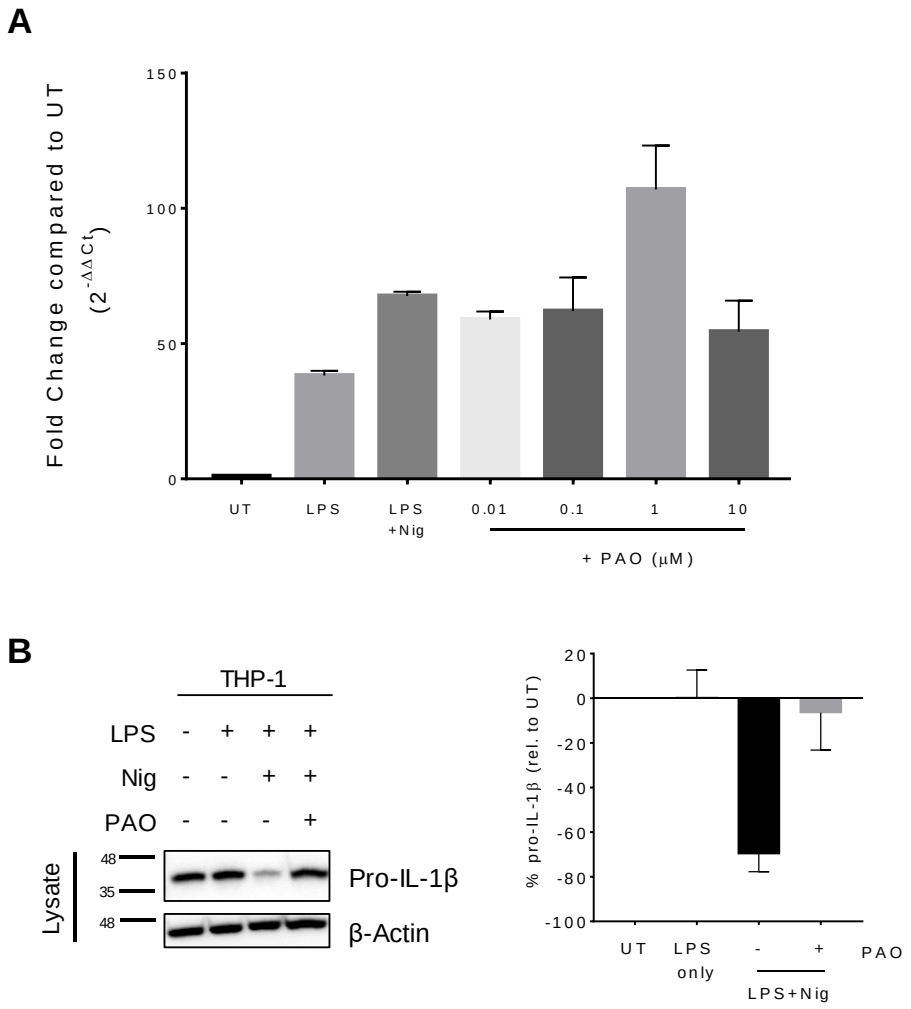

Figure 4-1: Post-LPS PAO treatment does not affect pro-IL-1β transcription and translation

*BMDMs (**A**) or THP1DMs (**B**) were primed with 1 μg/ml LPS for 3 hours followed by treatment with PAO (0.01 – 10 μM) in BMDMs and 1 μM PAO in THP1DMs for 30 minutes. Nigericin (10 μM) was then added for 1 hour to activate the NLRP3 inflammasome. **A** Quantitative PCR was carried out on the BMDM mRNA for IL-1β (n=1) and the fold change calculated with $2^{-\Delta\Delta Ct}$. All data represent the means ± standard error. **B** Lysate from THP1DMs was assessed for pro-IL-1β protein by immunoblot and densitometric analysis carried out. Actin was used as the loading control for the immunoblot. Representative immunoblot figure of n=3.*

4.3 PAO inhibits nigericin-induced inflammasome activation in THP1DMs

To assess the effect of PAO on nigericin-induced NLRP3 inflammasome activation, THP1DMs were initially treated with LPS (1 μg/ml) for 3 hours and then PAO (0.01 μM – 10 μM) for 30 minutes prior to the addition of nigericin (10 μM) for 1 hour. Figure 4-2A shows that PAO inhibited nigericin induced NLRP3 inflammasome activation demonstrated by the reduction in the IL-1β release in a concentration-dependent manner. Caspase-1 is a cysteine protease activated by the processing of the precursor form into two subunits, p20 and p10. These subunits interact and form the active caspase-1 which then cleaves the IL-1β precursor, pro-IL-1β, to the bioactive ~17kDa fragment (Thornberry et al., 1992). The p10 subunit of caspase-1 and the bioactive p17 fragment of IL-1β were released into the extracellular space upon inflammasome activation. The IL-1β ELISA measures IL-1β release but cannot distinguish between the precursor and the caspase-1 processed active form of IL-1β. Therefore, in order to assess the processing and release of both IL-1β and caspase-1 (thus establishing activation of the NLRP3 inflammasome), a western blot analysis was carried out on acetone-precipitated supernatant from the treatments. Both IL-1β and caspase-1 were processed and released into the supernatant by THP1DM and BMDMs in the presence of nigericin, but processing was inhibited when PAO was added (Figure 4-2C), suggesting that PAO is affecting inflammasome activation.

Another cytokine released as a consequence of inflammasome activation is IL-18. We observed that PAO inhibited the release of IL-18 in THP1DMs (Figure 4-2B), further confirming the inflammasome inhibitory function of PAO.

Apart from caspase-1, IL-1β and IL-18 processing and release, another hallmark of nigericin induced inflammasome activation is the induction of cell death (Cullen et al., 2015). We found that PAO inhibited nigericin-induced cytotoxicity in THP1DM cells as measured as the amount of lactate dehydrogenase (LDH) released (Figure 4-2D).

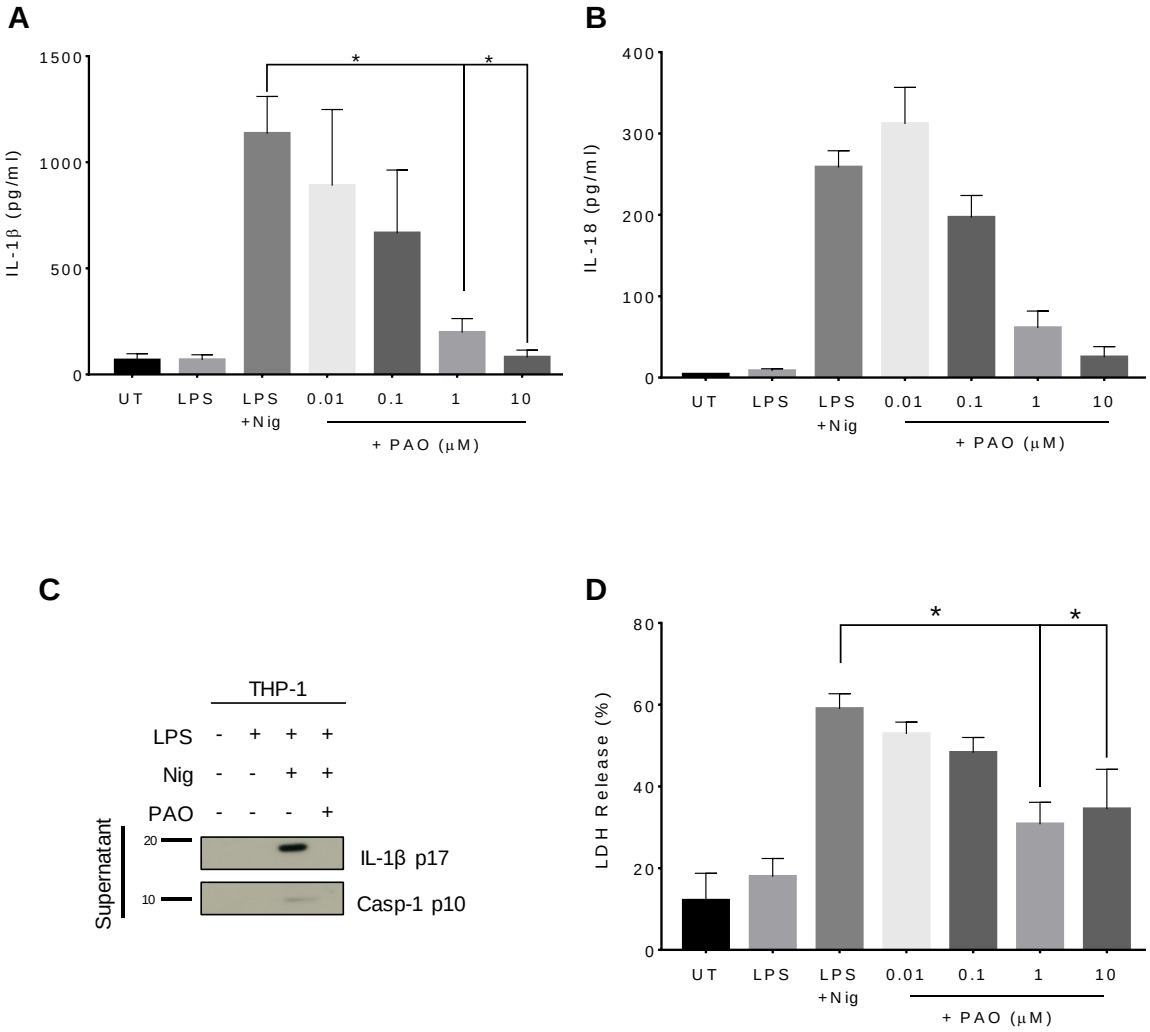

Figure 4-2: PAO inhibits nigericin-induced inflammasome function in THP1DM cells

THP1-derived macrophages were primed with 1 μg/ml LPS for 3 hours followed by treatment with PAO (0.01 – 10 μM) or vehicle for 30 minutes. Nigericin (10 μM) was then added for 1 hour to activate the NLRP3 inflammasome. ***A****. An ELISA of the supernatant was carried out to measure IL-1β release from THP1DMs.* ***B.*** *Human IL-18 was also measured by ELISA (n=2).* ***C.*** *Processing of both IL-1β and caspase-1 were assessed by immunoblot in THP1 macrophages with 1 μM PAO.* ***D****. LDH measurement was carried out on THP1 macrophages supernatants to assess cell death. All data represent the means ± standard error (SEM). Statistical analysis was carried out by comparing each of the inhibitor treatment conditions to the LPS+Nig column using ordinary one-way ANOVA with the Dunnett test. Level of significance: *p ⩽0.05 (One-Way ANOVA) (n=3).*

4.4 Nigericin-induced inflammasome activation is inhibited by PAO in BMDM cells

To validate the effect of PAO on nigericin-induced NLRP3 inflammasome activation observed in THP1DMs, mouse cells (BMDMs) were initially treated with LPS (1 μg/ml) for 3 hours and then PAO (10 μM - 0.01 μM) for 30 minutes prior to the addition of nigericin (10 μM) for 1 hour. PAO inhibited nigericin-induced NLRP3 inflammasome activation in a concentration-dependent manner shown as the reduction in the IL-1β release (Figure 4-3). PAO drastically reduced IL-1β release in BMDMs but at a lower concentration of 0.1 μM compared to THP1DMs (Figure 4-3A). In addition, to assess the processing and release of IL-1β and caspase-1, immunoblot analysis was carried out on the acetone-precipitated supernatant. BMDMs were treated with LPS for 3 hours followed by 30 minutes with 0.1 μM then 1 hour with nigericin. We observed that both IL-1β and caspase-1 were processed and released into the supernatant in the presence of nigericin but inhibited when PAO was added (Figure 4-3B).

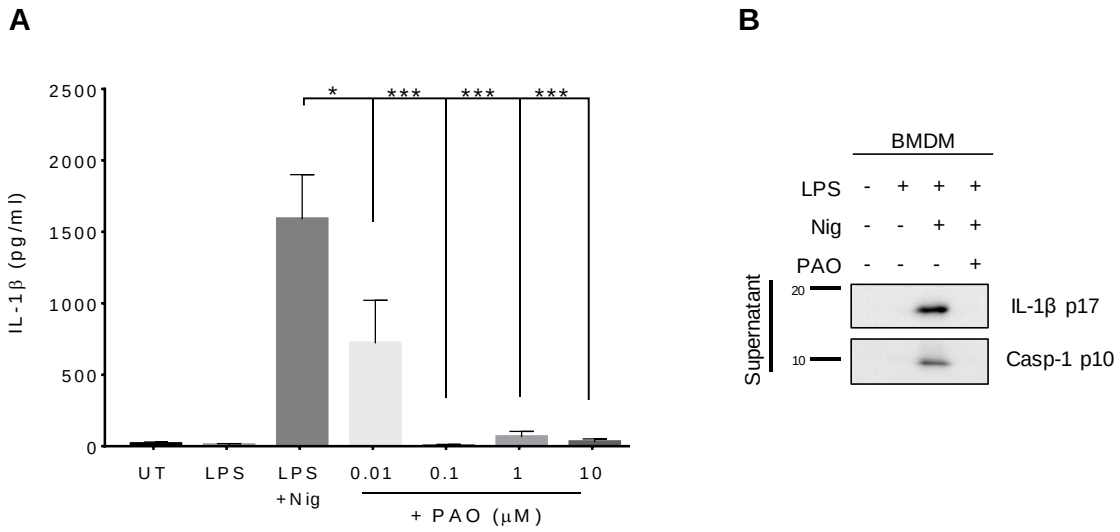

Figure 4-3: PAO inhibits nigericin-induced inflammasome function in BMDMs

*BMDMs were primed with 1 μg/ml LPS for 3 hours followed by treatment with PAO (0.01 – 10 μM) or (0.1 μM PAO for immunoblot analysis). Nigericin (10 μM) was then added for 1 hour to activate the NLRP3 inflammasome. An ELISA of the supernatant was carried out to measure IL-1β release (**A**). Processing of both IL-1β and caspase-1 were assessed by immunoblot (**B**) (n=3). All data represent the means ± standard error (SEM). Statistical analysis was carried out by comparing each of the inhibitor treatment conditions to the LPS+Nig column using ordinary one-way ANOVA with the Dunnett test. Level of significance: *p ⩽0.05; ***p ⩽0.001 (n=3).*

4.5 PAO inhibits MSU-mediated NLRP3 inflammasome activation in human and murine macrophages

The deposition of soluble urate crystals in joints and connective tissue is the main cause of gout (Martillo et al., 2014). Urate crystals deposited into these sites lead to a localised inflammatory response as a result of NLRP3 inflammasome activation. Monosodium urate (MSU) crystals function like soluble urate as an NLRP3 inflammasome activator *in vitro* by mediating lysosomal destabilisation (Shi et al., 2014).

To assess the effect of PAO on MSU-induced NLRP3 inflammasome activation, THP1DMs were initially treated with LPS (1 μg/ml) for 3 hours (signal 1) and then PAO (0.01 μM – 10 μM) for 30 minutes prior to the addition of MSU crystals (200 μg/ml) for 6 hours. PAO inhibited MSU-induced NLRP3 inflammasome activation in a concentration-dependent manner demonstrated by the reduction in IL-1β release in both THP1DMs (Figure 4-4A) and BMDMs (Figure 4-4B).

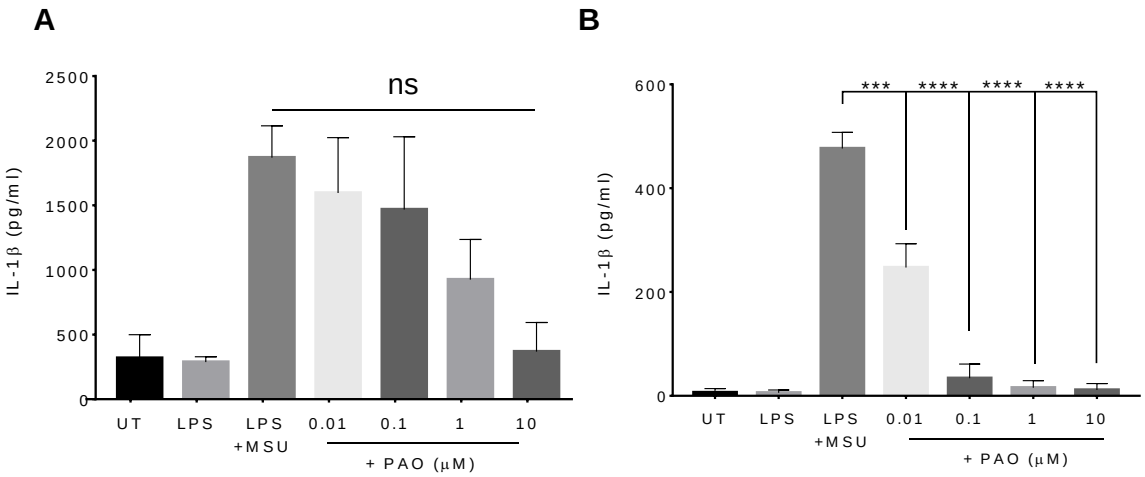

Figure 4-4: PAO inhibits MSU-induced inflammasome function in THP1DMs and BMDMs

*THP1DMs and BMDMs were primed with 1 μg/ml LPS for 3 hours followed by treatment with PAO (0.01 – 10 μM) or 0.1 μM PAO (for western blot analysis). MSU crystals (200 μg/ml) was then added for 6 hours to activate the NLRP3 inflammasome. An ELISA of the supernatant was carried out to measure IL-1β release in THP1DMs (**A**) and BMDMs (**B**). All data represent the means ± standard error (SEM). Statistical analysis was carried out by comparing each of the inhibitor treatment conditions to the LPS+MSU column using ordinary one-way ANOVA with the Dunnett test. Level of significance: ***p ⩽0.001; ****p ⩽0.0001; ^{ns}p> 0.05 (n=3).*

4.6 PAO abrogates poly(dA:dT)-mediated AIM2 inflammasome activation in human and murine macrophages

The AIM2 inflammasome is formed in response to cytoplasmic double-stranded DNA from viral and bacterial sources (Hornung et al., 2009). Experimentally, a double-stranded DNA agonist of the AIM2 inflammasome is poly(deoxyadenylic-deoxythymidylic) or poly(dA:dT). Poly(dA:dT) is a repetitive synthetic double-stranded DNA sequence that, when transfected into cells, is directly detected by AIM2 via its HIN-200 DNA binding domain and leads to the recruitment of ASC via the PYRIN domain. The oligomerisation and formation of the ASC protein speck subsequently take place recruiting pro-caspase-1 via its CARD domain and leading to cytokine release (IL-1β) (Hornung et al., 2009).

To assess the effect of PAO on poly(dA:dT)-induced AIM2 inflammasome activation, THP1DMs and BMDMs were initially treated with LPS (1 μg/ml) for 3 hours (signal 1) and then PAO (0.01 μM – 10 μM) for 30 minutes prior to the transfection of poly(dA:dT) (1 μg/ml with Lipofectamine 2000; signal 2) for 6 hours. PAO inhibited poly(dA:dT) induced AIM2 inflammasome activation in THP1DMs (Figure 4-5A) and BMDMs (Figure 4-5B). Cell death induced by poly(dA:dT) treatment in THP1DMs is also inhibited by PAO (Figure 4-5C), although this is not statistically significant.

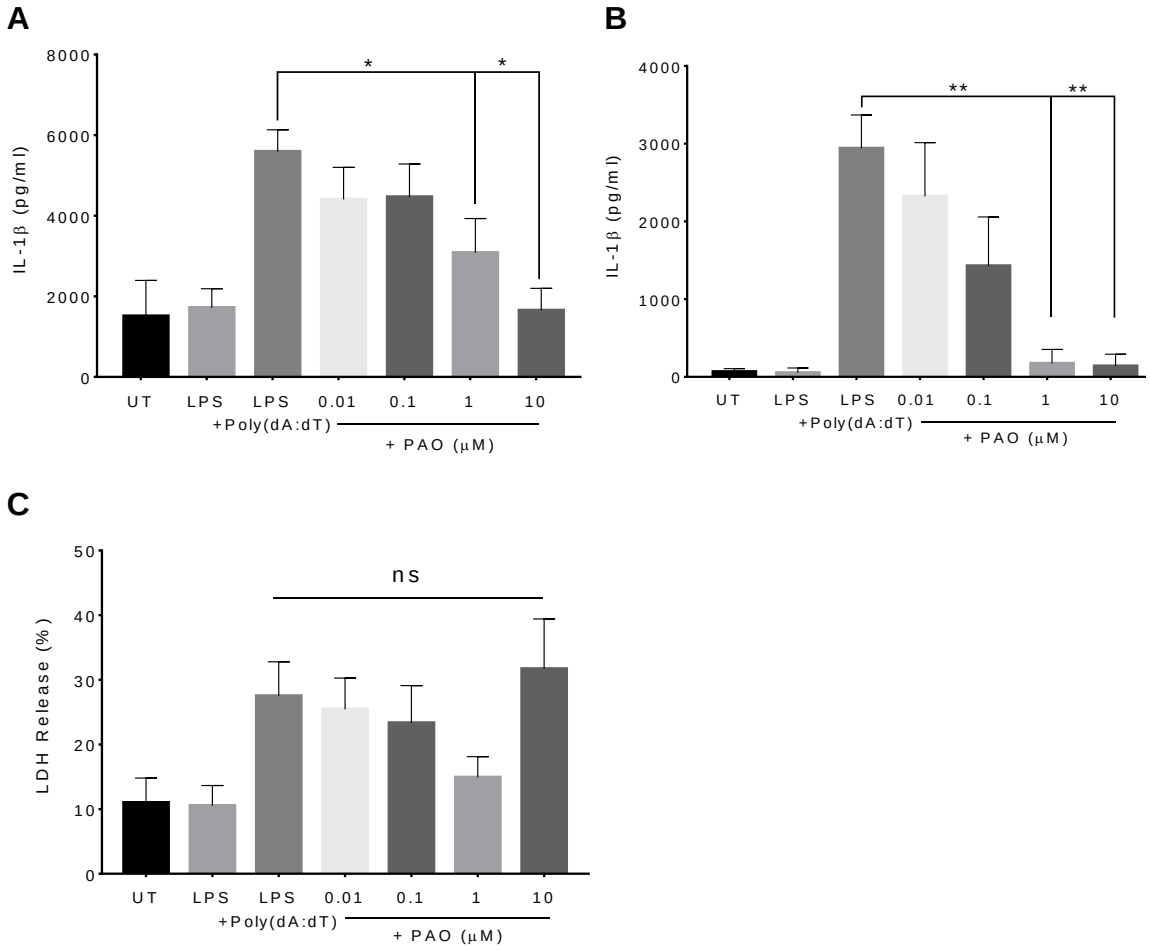

Figure 4-5: PAO inhibits poly(dA:dT) –induced AIM2 inflammasome function in THP1DMs and BMDMs

*THP1DMs and BMDMs were primed with 1 µg/ml LPS for 3 hours followed by treatment with PAO (0.01 – 10 µM) or vehicle (DMSO) for 30 minutes. Poly(dA:dT)(1 µg/µl) was transfected (1 µl Lipofectamine 2000) into the cells for 6 hours to activate the NLRP3 inflammasome. An ELISA of the supernatant was carried out to measure IL-1β release from THP1DMs (**A**) and BMDMs (**B**). LDH measurement was carried out on THP1 macrophages supernatants to assess cell death (**C**). All data represent the means ± standard error (SEM). Statistical analysis was carried out by comparing the mean of each of the inhibitor treatment conditions to the LPS+Poly(dA:dT) column using ordinary one-way ANOVA with the Dunnett test. Level of significance: *p ⩽0.05; **p ⩽0.01; ^{ns}p>0.05 (n=3).*

4.7 PAO abrogates S. *typhimurium*-mediated inflammasome activation in human and murine cells

Salmonella enterica serovar typhimurium (S. *typhimurium*) is a Gram-negative bacterium that is pathogenic (Coburn et al., 2007). Upon S. *typhimurium* infection, NLRP3 and NLRC4 have been shown to co-localise to form a functional inflammasome (Man et al., 2014) leading to IL-1β processing and release.

To assess the effect of PAO on S*. typhimurium*-induced inflammasome activation, we carried out treatment of THP1DMs with S. *typhimurium* overnight. PAO was added 30 minutes prior to treatment freshly cultured S. *typhimurium*. We show that in THP1DMs, S. *typhimurium* induced inflammasome activation shown by the release of IL-1β. The addition of PAO resulted in inhibition of IL-1β release (Figure 4-6A). As S. *typhimurium* is implicated in activation of the NLRC4 inflammasome, we sought to investigate the effect of PAO on the NLRC4 inflammasome by removing any effect that is a result of NLRP3 inflammasome activation. Therefore, the NLRP3 receptor protein was inhibited by the NLRP3-specific inhibitor MCC950 (Perera et al., 2018). We observed that inhibition of the NLRP3 receptor resulted in a drastic decrease in overall IL-1β release (Figure 4-6A) and the decrease was further augmented in the presence of PAO. S. *typhimurium* treated THP1DMs did not show marked decrease in LDH release (a measure of cytotoxicity) despite PAO or MCC950 treatment (Figure 4-4C), but a higher concentration of PAO (10 μM) induced cytotoxicity.

With this observation in IL-1β release and cell death inhibition as a result of MCC950 treatment, we sought to further validate this data by using Nlrp3-deficient BMDMs in our investigation. Nlrp3-deficient BMDMs had a completely attenuated response to S. *typhimurium* infection compared to wild-type BMDMs demonstrated by IL-1β release (Figure 4-6C). PAO, as in MCC950 untreated THP1DMs, inhibited IL-1β release in wild-type BMDMs (Figure 4-4B), suggesting that S. *typhimurium* infection in both human and mouse macrophages induces IL-1β release that is PAO sensitive. Furthermore, *S. typhimurium* treatment in wild-type BMDMs appears to have a higher basal release of LDH (Figure 4-4D) compared to Nlrp3-deficient BMDMs. Therefore, in both THP1DMs and BMDMs, the addition of S. *typhimurium* did not result in increased LDH release implying that this preparation of bacteria was not inducing cell death.

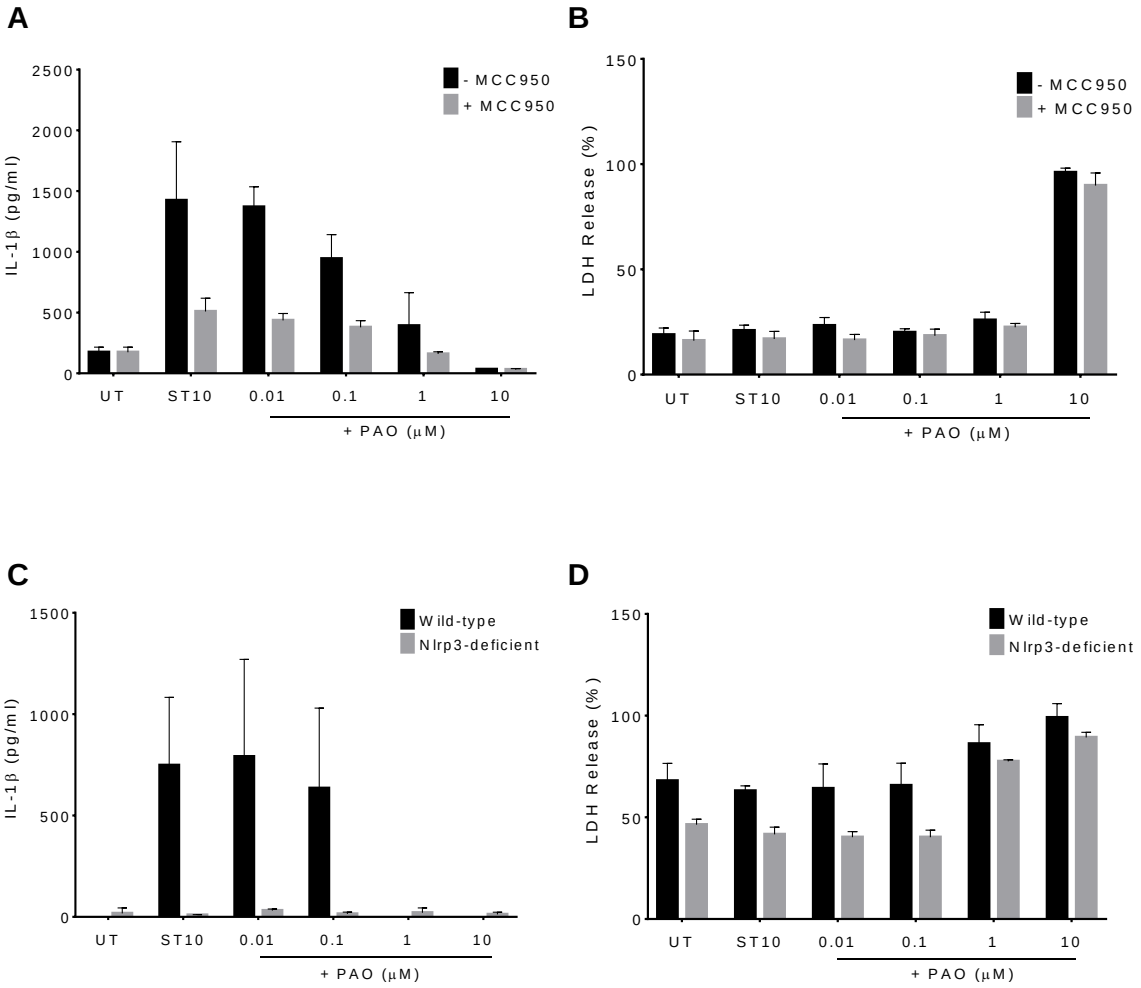

Figure 4-6: PAO abrogates S. *typhimurium*-mediated inflammasome activation in *human and murine macrophages*

Wild-type THP1-derived macrophages were incubated with S. typhimurium (ST10; MOI10) for 1 hour in the presence or absence of MCC950 (10 μM) ± PAO (0.01 – 10 μM) 30 minutes prior. Gentamycin (100 μg/ml) was then added overnight. ***A*** *An ELISA of the culture media (supernatant) was carried out to measure IL-1β release.* ***B*** *LDH measurement was carried out to assess cell death. Wild-type or Nlrp3-deficient BMDMs were incubated with S. typhimurium (MOI10) for 1 hour (10 μM) ± PAO (0.01 – 10 μM) 30 minutes prior. Gentamycin (100 μg/ml) was then added overnight.* ***C*** *ELISA of IL-1β release and* ***D*** *LDH measurement. (n=2).*

4.8 PAO treatment of the MSU-induced peritonitis mouse model

MSU crystals are good particulate activators of the NLRP3 inflammasome *in vitro* by causing the release of IL-1β after treatment. MSU crystals have also been used as the go-to agonist for NLRP3 in studying the *in vivo* relevance of inflammasome activation. Injection of MSU crystals into the peritoneal cavity has been shown to lead to peritoneal infiltration of immune cells, particularly neutrophils, due to an increase in IL-1β levels (Spalinger and Scharl, 2018, Chen et al., 2006). Therefore, to assess the effect of PAO in an *in vivo* setting, in-house made MSU crystals were injected into the peritoneal cavities of C57BL/6 mice in the presence or absence of PAO. We initially injected mice with 1 mg/kg PAO dissolved in ethanol (0.05% in PBS) or DMSO (0.05% in PBS) to assess if PAO (ethanol vs. DMSO) would affect immune cells infiltration. PBS was used as the control. Using flow cytometry, we observed no apparent effect of PAO or the respective vehicles on the ratio of dendritic cells (Figure 4-7A), macrophages (Figure 4-7B), monocytes (Figure 4-7C) and neutrophils (Figure 4-7D). The total number of peritoneal cells (total PLF cells) (Figure 4-7E) was slightly reduced in the presence of the vehicle and/or PAO. The flow cytometry gating strategies used to measure the population of dendritic cells (Figure 7-1), macrophages (Figure 7-2), monocytes (Figure 7-3) and neutrophils (Figure 7-4) from the peritoneal lavage are shown in Appendix 1.

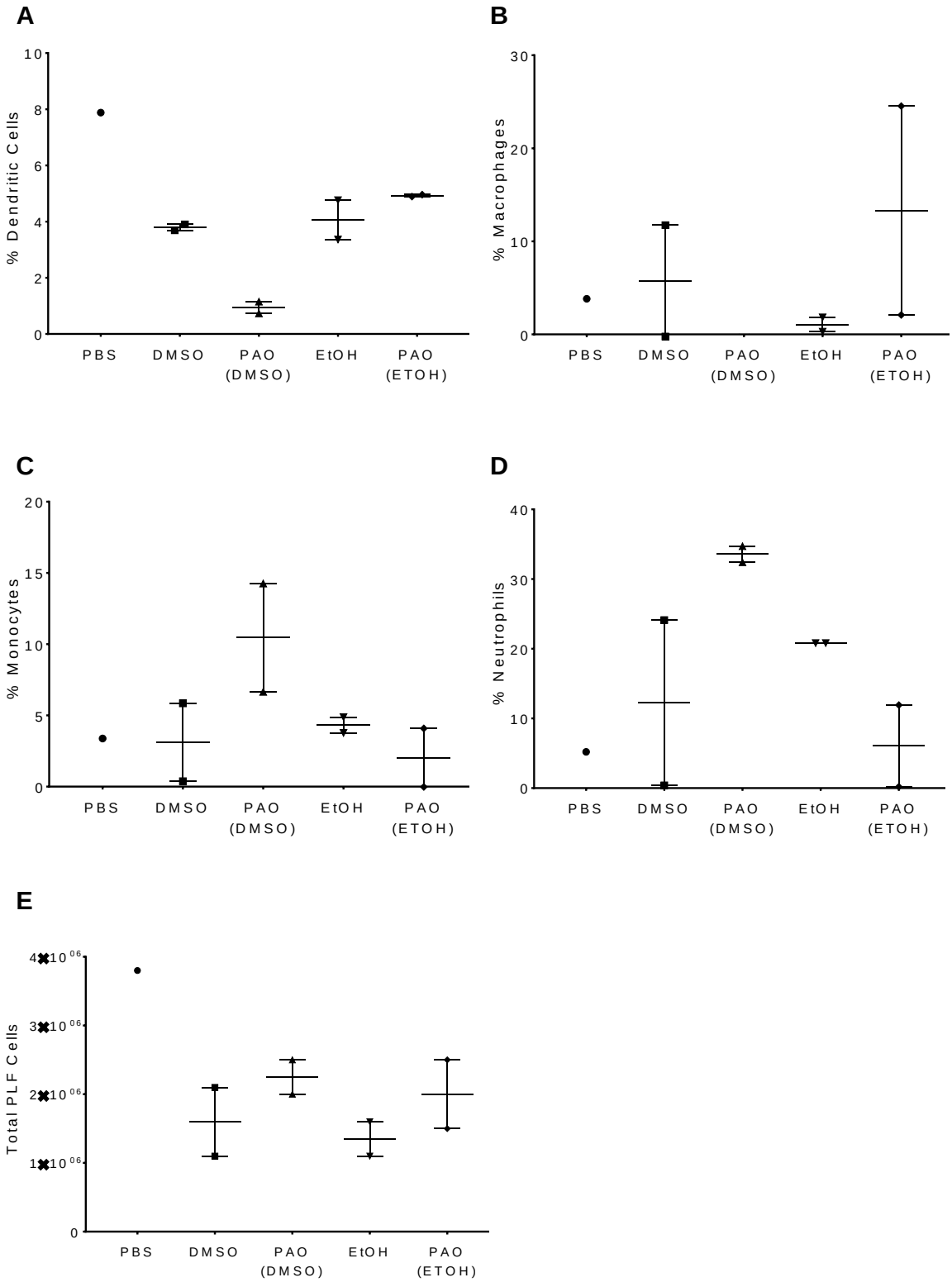

Figure 4-7: Testing DMSO and ethanol with PAO as activators of peritonitis
*Two C57BL/6 mice underwent an intraperitoneal injection with 1 mg/kg PAO or the corresponding ethanol or DMSO concentration for 2 hours. The mice were sacrificed, and the peritoneal cells were harvested by peritoneal lavage and flow cytometry was used to identify dendritic cells (**A**), macrophages (**B**), monocytes (**C**) and neutrophils (**D**) present within the peritoneal cavity. The number of live peritoneal lavage cells was counted as well (**E**) (n=1).*

We observed that PAO dissolved in DMSO had a slight pro-inflammatory effect in the peritoneal cavity by a slight increase in neutrophils compared to PAO dissolved in ethanol (Figure 4-7D). Therefore, we used ethanol as the vehicle of choice in the peritonitis treatment. The mice were injected intra-peritoneally with PAO at 1 mg/kg with a final vehicle concentration of 0.05% ethanol for 2 hours. MSU crystals (3.5 mg) were then injected into the mice for 5 hours prior to sacrifice. Cells collected by peritoneal lavage were then analysed by flow cytometry to assess infiltration of immune cells and cell viability. There were no differences between the various treatments in regard to the proportion of immune cells present within the peritoneal cavity (Figure 4-9). Interestingly, MSU crystals did not induce significant peritonitis; therefore, this implied that our intended MSU-induced peritonitis induction was unsuccessful possibly due to defective MSU crystals. The flow cytometry gating strategies used to measure the population of dendritic cells (Figure 7-5), macrophages (Figure 7-6), monocytes (Figure 7-7) and neutrophils (Figure 7-8) from the peritoneal lavage are shown in Appendix 1. Unfortunately, this experiment was not revisited due to time constraints and lack of access to mice.

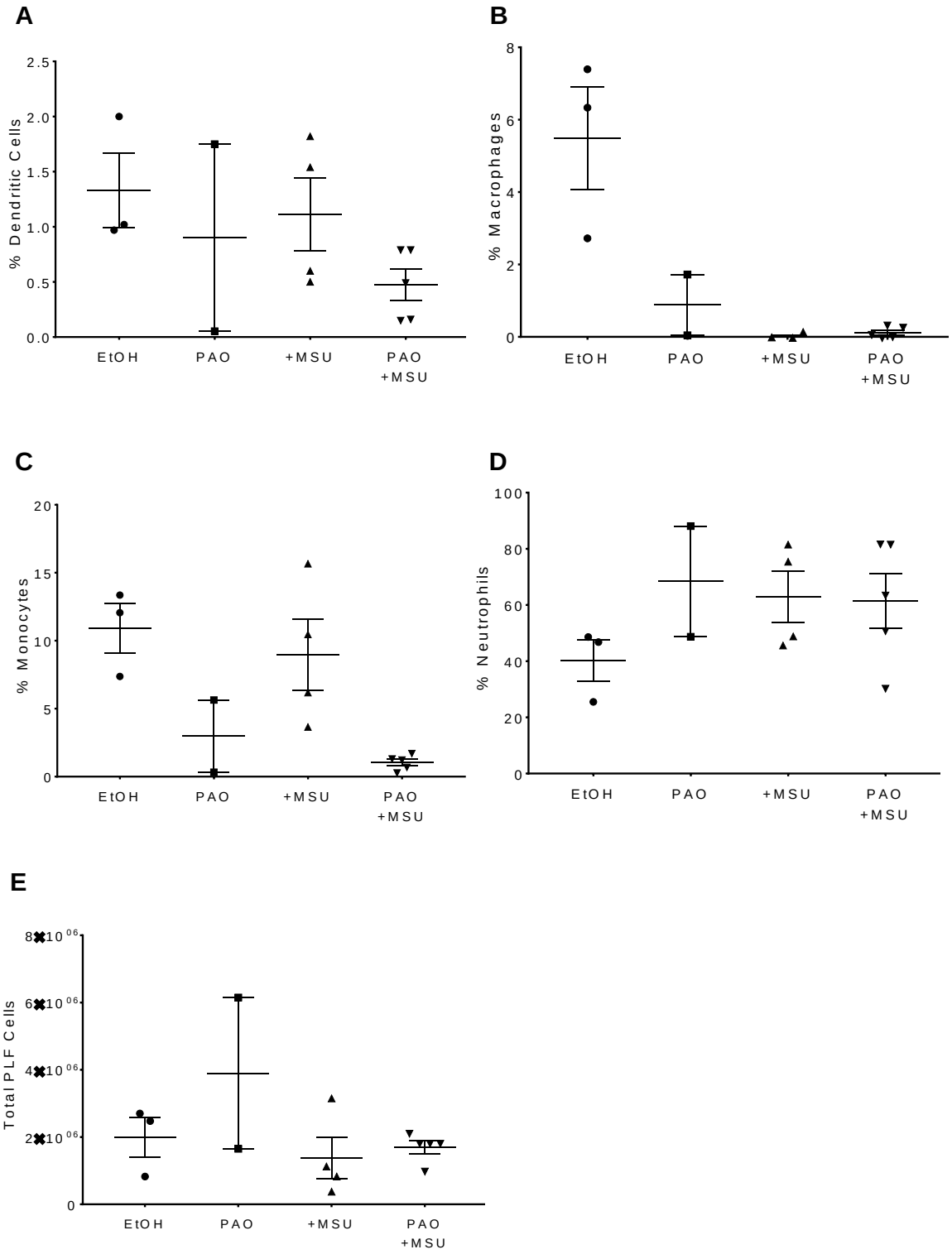

Figure 4-8: PAO treatment of the MSU-induced peritonitis mouse model
*Two/three C57BL/6 mice underwent an intraperitoneal injection with 1 mg/kg PAO or the corresponding ethanol concentration for 2 hours. MSU (3.5 mg) was then injected for another 5 hours. The mice were sacrificed, and the peritoneal cells were harvested by peritoneal lavage and flow cytometry was used to identify dendritic cells (**A**), macrophages (**B**), monocytes (**C**) and neutrophils (**D**) present within the peritoneal cavity. The number of live peritoneal lavage cells were counted as well (**E**) (n=1).*

4.9 PAO attenuates ASC oligomerisation and speck formation

Apart from cytokine release and cell death, the oligomerisation of the adaptor protein ASC is another hallmark of inflammasome activation (Fernandes-Alnemri et al., 2007, Lin et al., 2015, Khare et al., 2016, Dick et al., 2016). Upon detection of a "danger" signal, the NLRP3 receptor oligomerises and subsequently leads to the recruitment and oligomerisation of ASC (Dick et al., 2016)**.** The aggregation of ASC can be visualised as a single intracellular perinuclear speck by immunofluorescence microscopy. Therefore, THP1DM were cultured on microscope chamber slides and treated with LPS (1 μg/ml) for 3 hours and PAO (1 μM) for 30 minutes followed by 45- 60 minutes of nigericin (10 μM) treatment. The cells were fixed with 1-2% paraformaldehyde and stained with an anti-ASC antibody and a fluorophore-conjugated secondary antibody and then ASC was visualised by confocal microscopy. The formation of a single speck per cell was inhibited by PAO treatment in THP1DMs as there were less visible specks (Figure 4-9A). The percentage of cells containing an ASC speck was assessed by counting the number of specks and nuclei and calculating the percentage for 100 cells. A significant ($p=0.0047$) reduction in the number of specks was observed in the presence of PAO compared to its absence (Figure 4-9A) indicating that the PAO target is upstream of caspase-1 activation and affects ASC function.

An ASC oligomerisation assay was then carried out involving the treatment of cell lysate with the cross-linker disuccinimidyl suberate (DSS). Disuccinimidyl suberate is a cross-linker that reacts with amine groups to chemically cross-link interacting proteins (Khare et al., 2016). Following crosslinking, the cross-linked

product was analysed by immunoblot for the formation of multimers of the target protein. THP1DMs were treated with LPS (1 μg/ml) for 3 hours and PAO (1 μM) for 30 minutes followed by 60 minutes of nigericin (10 μM) treatment and the cells were harvested, lysed and DSS treated according to Khare *et al.* (2016) (Khare et al., 2016). An immunoblot shows that in the absence of PAO, nigericin treatment led to the formation of various multimers of the ASC protein (Figure 4-9B). PAO treatment, on the other hand, inhibited the formation of ASC multimers, suggesting that PAO targets a protein involved in allowing CARD-CARD and PYRIN-PYRIN domain interactions.

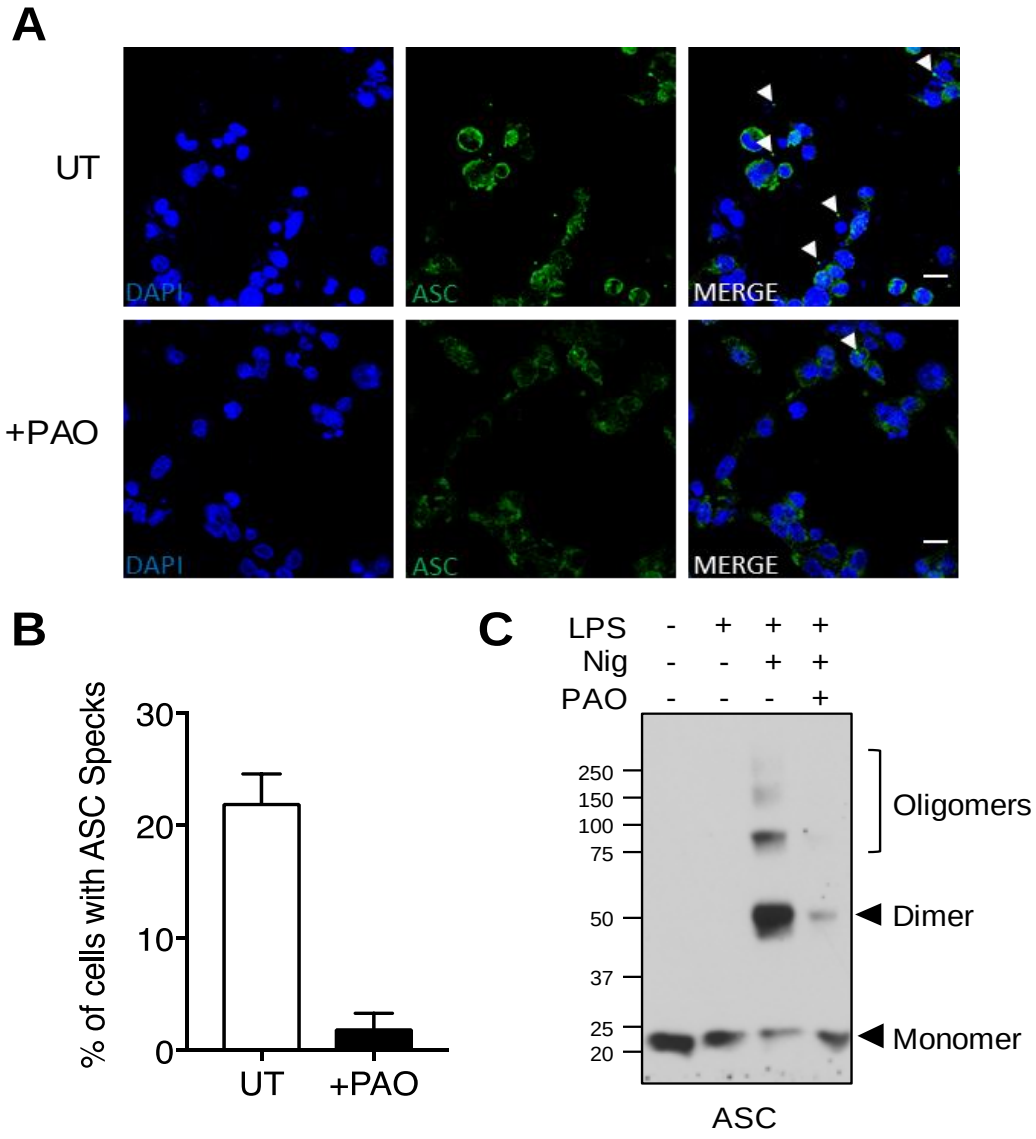

Figure 4-9: PAO inhibits ASC oligomerisation and speck formation

*THP1DMs were primed and treated with PAO (1 µM) for 30 minutes. Nigericin (10 µM) was then added for 30-45 minutes. Cells were fixed (2% paraformaldehyde) and immune-stained with an anti-ASC antibody (N-15-R) and anti-Rabbit Alexa fluor-488-conjugated secondary antibody then visualised by confocal microscopy (magnification 100X, scale bar 20 µM) (**A**). **B** The number of ASC specks per nucleus (DAPI stained) were counted and calculated as a percentage. All data represent the means ± standard error (SEM) (n=3). Two-tailed paired t-test was carried out with Gaussian distribution assumed (parametric test)* Level of significance**p≤0.01. DSS-mediated crosslinking of ASC monomers was carried out and analyzed by immunoblot (**C**). Representative figures of n=3.*

4.10 PAO treatment inhibits nigericin-induced ASC dephosphorylation

Previous work appears to suggest that the activation of the NLRP3 inflammasome pathway yields a variable tyrosine phosphorylation profile where there is overall phosphorylation and dephosphorylation of intracellular proteins (Hoyt et al., 2016). To determine the global tyrosine phosphorylation, immunoblot analysis of treated THP1DMs was carried out. Four conditions were assessed, untreated, LPS alone, LPS+nigericin and LPS+nigericin with 1 µM PAO. PAO treatment resulted in inhibition of global tyrosine dephosphorylation as observed in the OVN treatment (Figure 4-10A). The global tyrosine phosphorylation state of proteins increased in THP1DMs following LPS treatment. Nigericin treatment resulted in an overall reduction in phosphotyrosine whereas the addition of PAO prior to nigericin treatment inhibited the global tyrosine dephosphorylation as shown, thereby implicating that PAO exerts its inflammasome inhibitory effect by inhibiting PTPs.

Since we observed that PAO affects both ASC oligomerisation and global tyrosine dephosphorylation, we sought to assess the phosphorylation state of ASC at tyrosine residues. An immunoprecipitation of ASC and phosphotyrosine proteins was carried out from all the different treatment conditions and investigated by immunoblot. Immunoprecipitated ASC (ASC IP) was phosphorylated prior to nigericin treatment in both untreated and LPS only conditions (Figure 4-10B). Dephosphorylation of ASC took place upon nigericin treatment, but this effect was inhibited in the presence of PAO. The densitometric analysis of the western blot data revealed that the observed changes were not statistically significant as analysed by One-Way ANOVA (Dunnett Test) by comparing all other treatment conditions to the

untreated control. There is, however, a trend that can be observed where the addition of LPS causes a reduction in ASC phosphorylation and nigericin further augments this reduction. PAO treatment appears to prevent ASC dephosphorylation observed. On the other hand, when immunoprecipitation of phosphotyrosine proteins with a phosphotyrosine antibody (pTyr IP) was carried out, less ASC was immunoprecipitated in the nigericin treatment compared to the other conditions implicating a reduction in tyrosine phosphorylation following nigericin treatment (Figure 4-10B). Furthermore, IgG isotype control revealed that there was no non-specific binding of ASC (Figure 4-10B). Interestingly, we observed β-actin within the ASC IP.

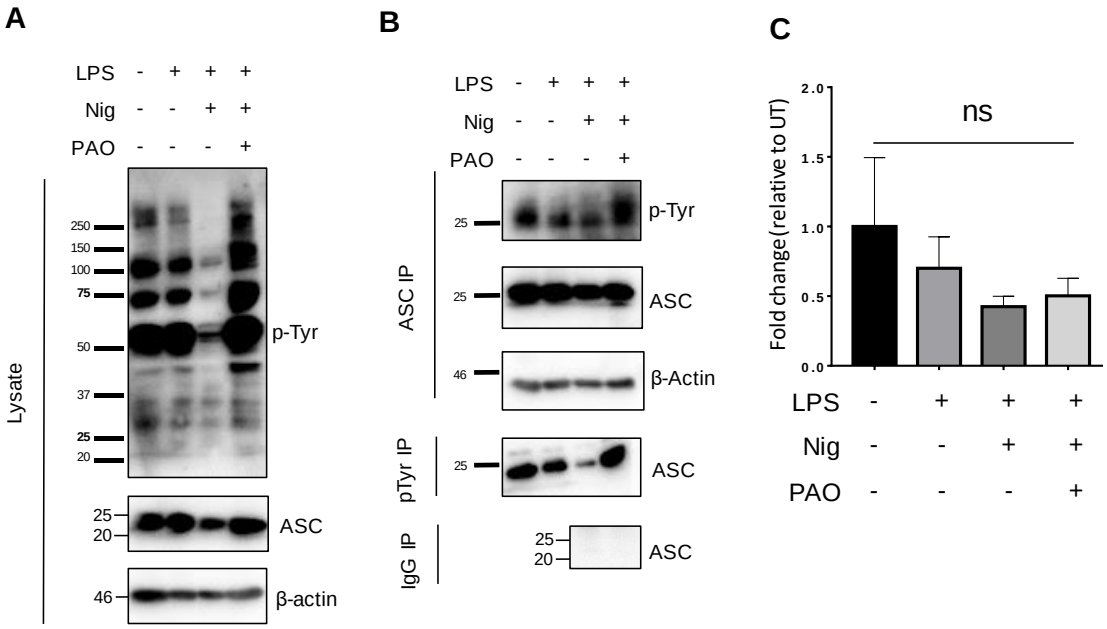

Figure 4-10: PAO treatment inhibits nigericin-induced overall tyrosine dephosphorylation and ASC tyrosine dephosphorylation

*THP1DMs were primed followed by treatment with PAO (1 µM) for 30 minutes. Nigericin (10 µM) was then added for 1 hour. Immunoblot analysis was carried out on whole lysate for tyrosine phosphorylation (**A**). Immunoprecipitation of ASC (ASC IP) and phosphotyrosine proteins (pTyr IP) was carried out then immunoblot was carried out for pTyr and ASC, respectively. ASC IP was then re-probed to assess immunoprecipitation success and loading control. Negative IgG control showed no non-specific ASC binding (**B**). Densitometric analysis of ASC-IP was carried out (**C**). Statistical analysis was carried out by comparing the mean of each of the inhibitor treatment conditions to the untreated (control) column using ordinary one-way ANOVA with the Dunnett test. Level of significance: not significant (p>0.05) (n=3). β-Actin was used as a loading control in total lysate. Representative figures of n=3.*

4.11 Discussion

We have shown that PAO is a potent inhibitor of nigericin, MSU crystals, poly(dA:dT) and *S. typhimurium* mediated activation of the inflammasome. PAO drastically inhibited the release of IL-1β and IL-18 in nigericin and MSU-induced NLRP3 inflammasome activation. Similarly, the AIM2 and NLRP3-NLRC4 (*S. typhimurium*) inflammasomes are inhibited by PAO treatment indicating that PAO affects proteins that are involved in the mechanisms of all the inflammasomes mentioned above. This is the first study to have demonstrated this effect of PAO on various inflammasome complexes. We also show that ASC, the adaptor protein, is dephosphorylated on tyrosine residue(s) upon activation of the inflammasome with nigericin but this dephosphorylation is inhibited by PAO. Furthermore, we have demonstrated that PAO inhibits global tyrosine dephosphorylation induced by nigericin, thus inhibiting inflammasome activation.

The measure of IL-1β and IL-18 release only sheds light on the final step of inflammasome activation but is limited on revealing where PAO is exerting its effect. Therefore, working upstream from IL-1β release, we sought to investigate the effect of PAO. We investigated the processing of pro-IL-1β and observed that nigericin treatment led to a reduction in pro-IL-1β due to increased processing into its bioactive 17kDa form. However, pro-IL-1β is restored in the presence of PAO. This observation, together with the qPCR in BMDMs, reveals that PAO has no effect on pro-IL-1β mRNA translation where PAO is added after LPS priming. In PAO treatment, we observed that pro-IL-1β levels were restored to the same level as the LPS only treatment with PAO. Furthermore, and importantly, PAO is also inhibiting

processing of IL-1β by caspase-1 implying that this PAO effect occurs further upstream of IL-1β processing.

We also observed that as with pro-IL-1β processing, pro-caspase-1 processing was also inhibited in the presence of PAO. Due to the nature of PAO to bind vicinal thiols from adjacent cysteines (Christina et al., 1992), the possible explanation for the inhibition of caspase-1 processing is the inhibitory effect of PAO on IL-1β-converting enzyme (ICE)-related caspases, such as caspase-1 (which undergoes auto-proteolysis (Elliott et al., 2009)) implicated by Takahashi et al (Takahashi et al., 1997). Before concluding that PAO was directly inhibiting caspase-1 function, we sought to investigate whether PAO was acting upstream of caspase-1. ASC oligomerisation and subsequent speck formation is required for pro-caspase-1 recruitment to the inflammasome and caspase-1 processing. Furthermore, Man *et al.* revealed that ASC speck formation was not affected by direct caspase-1 inhibition (Man et al., 2014). We observed that ASC oligomerisation and speck formation was inhibited by PAO, revealing that PAO-mediated inhibition of caspase-1 processing is due to inhibition of ASC oligomerisation, which consequently inhibits pro-caspase-1 recruitment to the inflammasome complex. This, therefore, reinforced the hypothesis that PAO is acting upstream of caspase-1. However, although it is possible that PAO could directly bind and inhibit caspase-1 function resulting in diminished IL-1β release, the inhibition of the upstream event of ASC oligomerisation and speck formation indicates that caspase-1 auto-processing is not activated in the first place as it is not recruited to form the inflammasome.

Hoyt *et al.* reported that tyrosine phosphatase inhibition, mediated by sodium orthovanadate, augmented ATP-induced inflammasome activation. In the same study, Hoyt and colleagues also reported that ethanol and small chain alcohol molecules activated tyrosine phosphatases, which led to the inhibition of the inflammasome (Hoyt et al., 2016). This is contradictory to what we have observed; where PAO mediated inhibition of global tyrosine dephosphorylation led to the inhibition of nigericin induced inflammasome function. We have shown that PAO-mediated inhibition of cytokine release is accompanied with global inhibition of tyrosine phosphatases. Although PAO and OVN are both broad-spectrum PTP inhibitors, their mechanisms of action differ. As previously stated, PAO exerts its inhibitory function by binding to vicinal thiols within the site of PTPs. On the other hand, OVN is similar in structure to phosphate and thus exerts its function by competitively occupying or masking the active site (Korbecki et al., 2012). As discussed in chapter 3, a number of factors could account for this observed disparity such as cell type, cell species and NLRP3 stimuli. Furthermore, OVN and PAO exert different inhibitory mechanisms which could account for the difference in effect on inflammasome activation. However, further work is required to fully dissect the mechanisms involved.

Alternatively, PAO has been reported to have non-PTP targets such as NADPH oxidase 2 (NOX2), calcineurin and RhoA GTPases that have been shown to affect IL-1β release. NOX2 is inhibited by PAO (Kazufumi et al., 2015, Doussiere et al., 1998, Cabec and Maridonneau-Parini, 1995) and regulates oxidative stress-induced NLRP3 inflammasome activation in murine neural tissue (Ma et al., 2017). The calcium and calmodulin-dependent serine/threonine phosphatase calcineurin

(CN) is inhibited by PAO in the bovine brain with an IC_{50} of 3-8 μM (Bogumil et al., 2000). Constitutively active calcineurin activates the NLRP3 inflammasome in CN transgenic mice implying that calcineurin plays an inflammasome activation role in myocardial tissue (Bracey et al., 2013). RhoA GTPase possesses the vicinal thiols required for PAO mediated inhibition (Gerhard et al., 2003), however, RhoA GTPase has only been reported to be involved in the pyrin inflammasome (Park et al., 2016) and not the NLRP3 inflammasome. Although NOX2, calcineurin and RhoA GTPase are PAO-sensitive, inhibition by PAO does not account for the observed inhibition of both NLRP3 and AIM2 inflammasomes in this study. Further work would, however, be required to determine whether PAO inhibition of the aforementioned enzymes would affect the NLRP3 inflammasome by utilising specific inhibitors or genetic interventions against the above proteins in parallel with PAO treatments.

To determine whether this inhibitory effect could be repeated *in vivo*, we utilised an established MSU induced peritonitis mouse model (Chen et al., 2006). However, in our first attempt, we noticed that the MSU (made in-house) used did not lead to increased infiltration of immune cells as expected, implying defective MSU. Due to time constraints and mice availability, we were unable to revisit this experiment using commercially available MSU crystals, however, this would be a crucial experiment to carry out as this will provide relevance of the *in vitro* model of PAO inflammasome inhibition.

The overall tyrosine phosphorylation was reduced as a result of nigericin treatment but was restored in the presence of PAO. This is a vital observation in understanding tyrosine phosphorylation in inflammasome complex formation. With

the focus on tyrosine phosphorylation in the context of the inflammasome, the “PAO only” treatment condition was not carried out. However, this would be an experimentally crucial control and its lack is considered a caveat of this study.

ASC tyrosine phosphorylation at Y146 is a necessary post-translational modification that leads to the assembly of the inflammasome (Chung et al., 2016, Hara et al., 2013b). In these studies, the investigators showed that mutant variants of the ASC protein, where phenylalanine substituted tyrosine, results in attenuated ASC function and consequently inflammasome activation. Furthermore, when inhibitors of Syk, JNK and Pyk2 were used, they were able to attenuate inflammasome function, suggesting that tyrosine phosphorylation of ASC (at Y146) is required; however, ASC dephosphorylation has not been studied. We carried out an ASC immunoprecipitation (IP) experiment following treatment with PAO and assessed tyrosine phosphorylation. Interestingly, β-actin was present in the IP of ASC which could suggest ASC-β-actin interaction as previously shown where Filamentous actin (F-actin) interacts with NLRP3 and ASC in the presence of the protein LRRFIP2. This was shown to be enhanced in the presence of ATP and nigericin and demonstrated to facilitate the inhibition of caspase-1 activation by the protein FliI (Burger et al., 2016). However, we saw interaction prior to activation therefore more work would be required to elucidate ASC – actin interactions and the role actin plays in inflammasome complex regulation.

More importantly, we have shown that ASC tyrosine dephosphorylation takes place following inflammasome activation with nigericin. We observed that PAO inhibited ASC dephosphorylation, which suggests that tyrosine dephosphorylation is

also required for inflammasome activation. Since ASC forms a speck following inflammasome activation, it is conceivable that the reduction in immunoprecipitated phosphorylated ASC is due to less ASC available to immunoprecipitated since ASC is the speck. However, it has been shown that ASC can be immunoprecipitated following inflammasome activation in similar lysis and immunoprecipitation conditions as carried out in this study (Chung et al., 2016). Following densitometric analysis, we found that the decrease in ASC phosphorylation is not statistically significant, therefore, further work involving more precise methodologies is required to further determine the phosphorylation state of ASC following the treatments done here. A precise method to carry out this work would be mass spectrometry on immunoprecipitated ASC under various conditions to assess the phosphorylation state of tyrosine residues. This method will provide a much needed quantitative and precise read-out on the state of tyrosine phosphorylation on ASC.

In conclusion, with the aid of PAO, we have shown that global inhibition of PTPs (and therefore global tyrosine dephosphorylation inhibition) inhibits NLRP3 and AIM2 inflammasome activation. Furthermore, we report that ASC tyrosine dephosphorylation is required for normal nigericin induced NLRP3 inflammasome activation.

5. Identification of the tyrosine phosphorylation sites on ASC

5.1 Introduction

According to Hara *et al.* & Chung *et al.*, phosphorylation at tyrosine (Y) 144/146 is an essential post-translational modification of ASC that leads to its ability to oligomerise and thus form a functional inflammasome with the receptor and the effector caspase-1 (Hara et al., 2013b, Chung et al., 2016). Using the tyrosine phosphatase inhibitor PAO, we have shown in Chapter 4 that the dephosphorylation of tyrosine plays a crucial role in the regulation of the inflammasome as a whole and ASC specifically. We, therefore, next sought to identify the putative tyrosine residues that are required for the function of human ASC.

To this aim, tyrosine residue identification was carried out based on the following criteria:

- Conservation between human and mouse (Figure 5-1A)
- Predicted phosphorylation using the phosphonet.ca web tool (Figure 5-1B).

Figure 5-1: ASC tyrosine residues selection criteria

*Tyrosine residues were selected based on the following criteria: **A**. Conservation between human and mouse **B**. Predicted phosphorylation based on the phosphonet.ca. The arrow (▼) indicates the identified tyrosine residues with surrounding amino acid sequence.*

With these criteria, the tyrosine residues identified were Y36, Y64, Y60 and Y137. Since previous publications have shown that the Y146 residue is essential for human ASC function, this residue was also included as an ASC functional control (Chung et al., 2016, Lin et al., 2015, Hara et al., 2013a). The identified residues and Y146 were subjected to site-targeted mutagenesis to the non-phosphorylatable phenylalanine (F). Both tyrosine and phenylalanine are aromatic and slightly hydrophobic, therefore, structurally and chemically similar but phenylalanine lacks a reactive hydroxyl group (Figure 5-2) (Betts and Russell, 2003). The similarity in structure is advantageous in such investigations because the overall structure of the protein is not drastically affected.

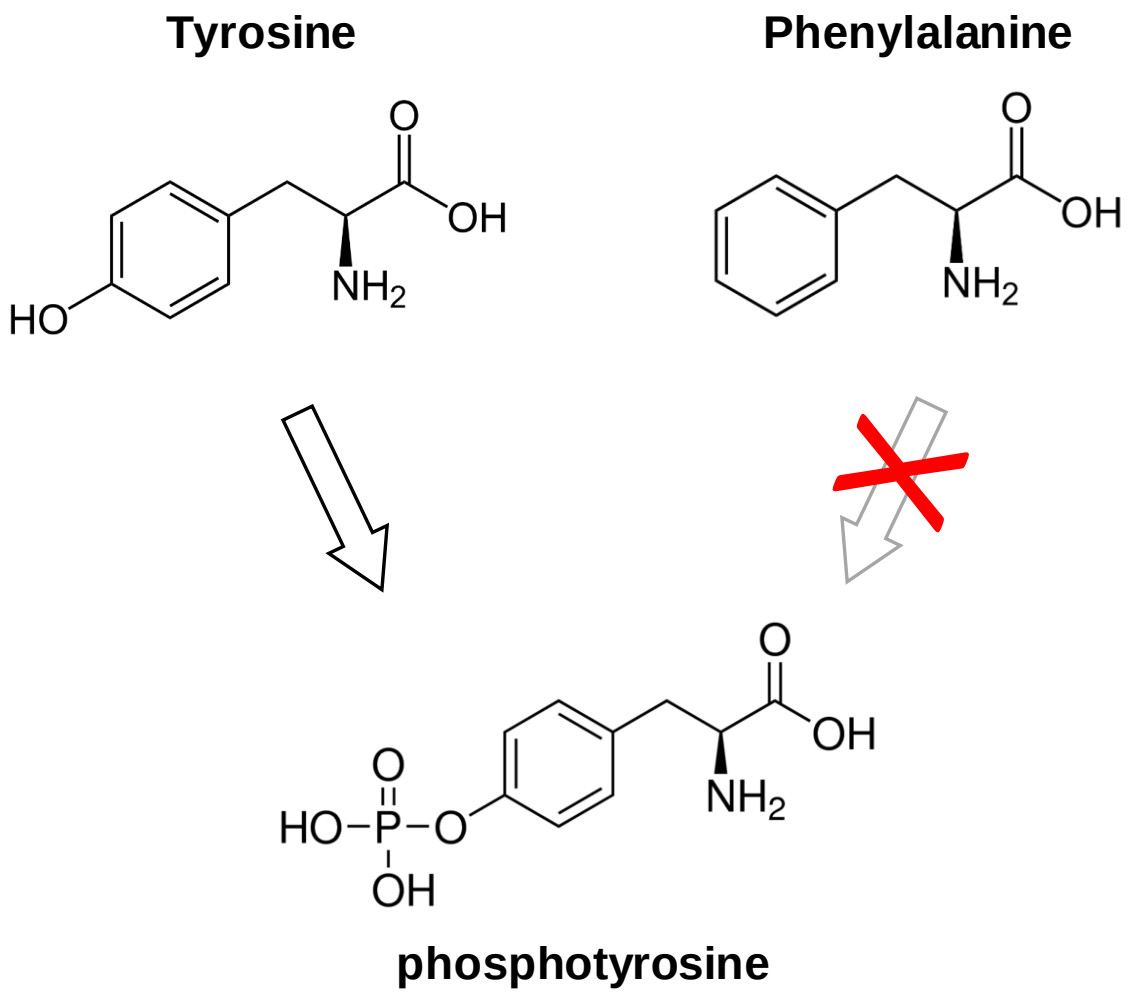

Figure 5-2: Comparing the structure of tyrosine and phenylalanine
Tyrosine (Y) possesses a hydroxyl (-OH) group on the benzene ring where the phosphoryl group is added during phosphorylation to generate phosphotyrosine. On the other hand, phenylalanine (F), although quite similar in structure to tyrosine, lacks the hydroxyl group required for phosphorylation and as such cannot generate phosphotyrosine.

5.2 Phosphorylation of tyrosine residues Y60, Y137 and Y146 are indispensable for NLRP3 inflammasome function

The selected tyrosine residues (Y36, Y60, Y64 & Y146) on ASC were mutated using the Q5 site-directed mutagenesis kit (NEB) in the pEF6-ASC-GFP plasmid (kindly provided by Dr Kate Schroder). The tyrosine codons (TAC or TAT) were substituted to phenylalanine (TTC or TTT, respectively) and mutational PCR was carried out according to manufacturer's (NEB) instructions. Following mutation, DH5α chemically competent *E. coli* were transformed with each of the five mutant ASC plasmids and a mini-prep was carried out and isolated plasmids were sent for sequencing (1st Base, Singapore). Figure 5-3 shows the validated mutant alignment of the ASC mutant against the wild-type. However, generation of the Y64F ASC mutant was unsuccessful using the Q5 site-directed mutagenesis kit and was not attempted again due to time constraints.

The human NLRP3 inflammasome was reconstituted in HEK293T cells by transfecting the human NLRP3-Flag, ASC-GFP (wild-type and mutant variants), pro-caspase-1 and pro-IL-1β. Following an incubation period of 48 hours, the supernatant and cell lysates were collected for ELISA and western blot analysis, respectively. The western blot confirmed that the different ASC variants were equally expressed in transfected HEK293T cells, as assessed by western blot. We found that the NLRP3 inflammasome that formed in the presence of the ASC mutants Y60, Y137 and Y146 had reduced IL-1β release compared to that containing the wild-type ASC. On the other hand, Y36 showed no significant difference in IL-1β release

compared to wild-type ASC (Figure 5-4). This implicates the Y60, Y137 and Y146 residues in normal NLRP3 inflammasome activation.

Figure 5-3: Validated ASC mutant sequence alignment
ASC mutant variants aligned with wild-type ASC validated by sequencing.

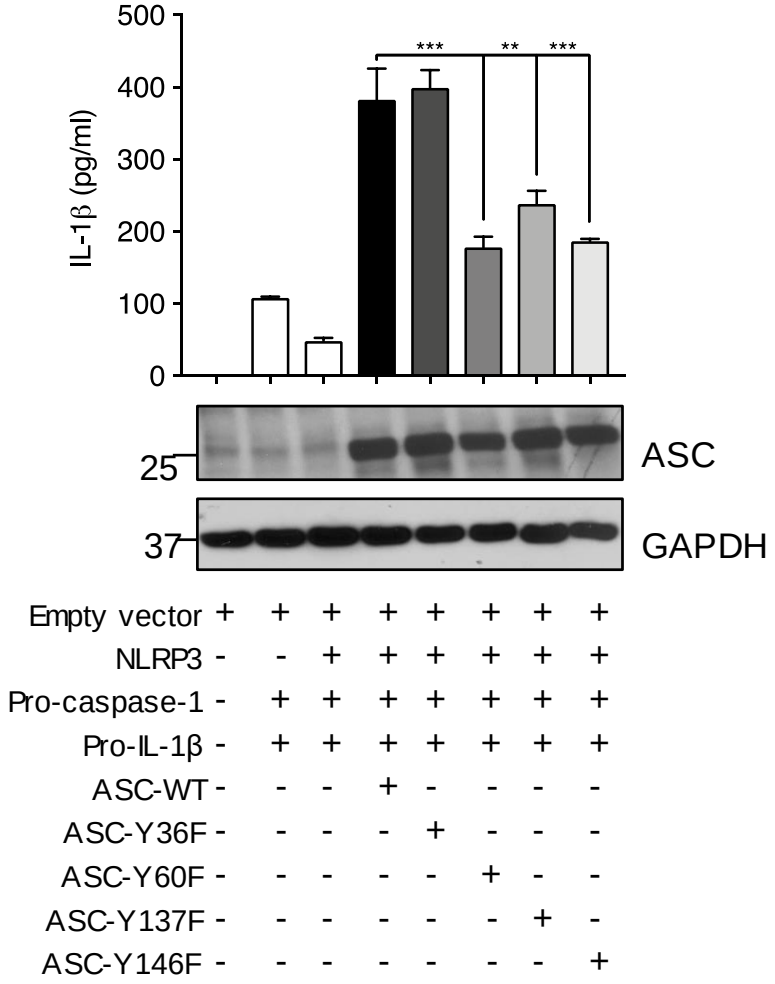

Figure 5-4: Y60, Y137 and Y146 ASC phosphorylation/dephosphorylation is required for NLRP3 inflammasome activation

*Site-directed mutagenesis of Y36, Y60, Y137 and Y146 to phenylalanine was carried out and the NLRP3 inflammasome was reconstituted in HEK293T cells by transfection of NLRP3, pro-caspase-1, IL-1β and validated ASC variants. IL-1β ELISA was carried out to measure the reconstituted NLRP3 inflammasome function. GAPDH was used as loading control for the immunoblot. . All data represent the means ± standard error (SEM) Statistical analysis was carried out by comparing the mean of each of the mutant transfection conditions to the ASC-WT column using ordinary one-way ANOVA with the Dunnett test. Level of significance: **p≤0.01, ***p≤0.001. Western blot representative images of n=3 (number of experiments).*

5.3 Discussion

We have demonstrated that successful mutagenesis of Y36, Y60, Y137 and Y146 to phenylalanine was carried out on the pEF6-ASC-GFP plasmid. An inflammasome function assay (IL-1β release by ELISA) in HEK293T cells showed that only the Y60, Y137 and Y146 residues in ASC caused an attenuation of the inflammasome function, while the Y36 mutation had no noticeable effect on inflammasome function.

Phosphorylation of Y146 in human ASC has previously been shown to be vital in inflammasome function, where it is targeted by Pyk2 upon inflammasome activation (Chung et al., 2016). Since the Y146 residue is located within the CARD domain of ASC, this suggests that it may be critical for CARD-CARD interactions, including ASC oligomerisation and caspase-1 recruitment. In this study, the identification of Y60 and Y137 tyrosine residues that require phosphorylation in ASC function is novel. Mutation of Y60 and Y137 in ASC to phenylalanine resulted in reduced IL-1β release in the HEK293T cells. Tyrosine 60 is the first residue identified within the pyrin domain (Figure 5-5) to require phosphorylation for ASC function. Since the tyrosine-to-phenylalanine mutation mimics dephosphorylation (while maintaining protein integrity), the reduction in IL-1β release suggests that dephosphorylation of Y60 inhibits PYRIN-PYRIN interactions which are NLRP3-ASC and ASC-ASC interactions. Therefore, the Y60 ASC mutant may have an attenuated ability to interact with NLRP3 and ASC oligomerisation, both required for normal inflammasome complex formation.

Mutation of ASC at the Y60, Y137 and Y146 residues revealed that tyrosine phosphorylation is required for the normal function of the ASC. Closer analysis of the structure of ASC as obtained from the PDB entry 2KN6 (Figure 5-5) revealed that Y60 and Y146 residues reside within the alpha helices of the protein, whereas Y36 and Y137 are located within the loop sections.

Our data showed that phosphorylation of Y36 was not required for NLRP3 inflammasome function but does not exclude this residue from being dephosphorylated (instead of requiring phosphorylation) since our mutational study was mimicking dephosphorylation or prevention of phosphorylation. Tyrosine 36 has been reported to form a hydrophobic patch with Y60 and Y64 which serves to anchor the H2-H3 loop to the rest of the protein. In an experiment performed to assess the interaction between ASC and its natural inhibitor PYD-only protein 1 (POP1), Srimathi *et al.* showed structural changes to the H2-H3 loop (such as the tyrosine-to-alanine mutation) resulting in the inability of ASC to interact with other proteins. This finding indicates that the Y36 residue, though not involved in direct PYRIN–PYRIN interactions, was necessary for the interaction to take place. Srimathi and colleagues further suggested that Y36 phosphorylation is required for the dissociation of POP1 from ASC, therefore, implying that phosphorylation of Y36 might serve as an inhibitory post-translational modification (Srimathi et al., 2008). Further work involving a constitutively phosphorylated mimic would be a valuable tool to use to investigate the extent to which this residue is required in mediating PYRIN-PYRIN interactions between ASC and other proteins, specifically NLRP3.

To further the understanding of ASC tyrosine phosphorylation, characterisation of these tyrosine mutants by assessing the ability of the mutants to interact with NLRP3 and form specks would be important. As mentioned before, an alternate strategy would be to substitute tyrosine residues with a constitutively phosphorylated tyrosine mimic to assess the effect of constitutive phosphorylation at these tyrosine residues on ASC function. This will then be followed by the generation of affinity peptides for each of the residues to allow pull-down and identification of targeting tyrosine phosphatases. Previous studies have used the amino acid glutamate to mimic constitutive phosphorylation (Kassenbrock and Anderson, 2004). However, glutamate is not a true mimic of phospho-tyrosine but rather is a better and more accurate mimic of phospho-serine/threonine (Makwana et al., 2017) and therefore would not be an appropriate mimic for constitutive tyrosine phosphorylation. Incorporation of a constitutively phosphorylated tyrosine would require technology that inserts a synthetic phosphotyrosine (pTyr) analogue into the full-length protein. This technology is currently not widely available; some reports have shown synthetic pTyr analogue insertion into peptides or by expansion of the genetic code in bacteria (Makwana et al., 2017, Hoppmann et al., 2017) both of which currently are in the early stages of development.

Another approach would be to generate phospho-specific antibodies against the tyrosine residues such as has been done for phospho-Y144 in murine ASC (Hara et al., 2013a). These antibodies will then serve as valuable tools in investigating the effect of kinase/phosphatase inhibitor libraries on ASC phosphorylation at these tyrosine residues, thus identifying candidate kinases/phosphatases targeting individual residues.

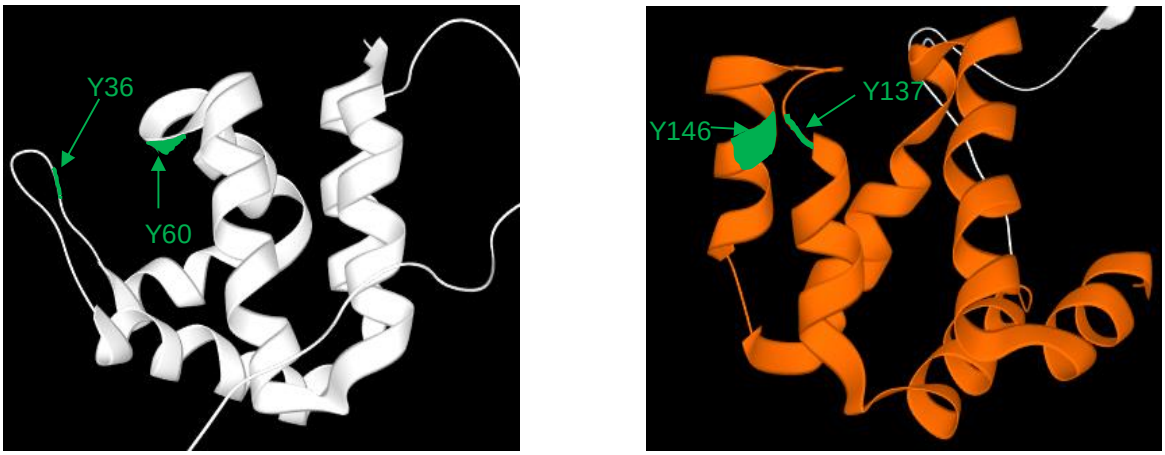

Figure 5-5: Localisation of the mutated tyrosine residues on ASC
With the aid of uniprot.org, the spatial locations of the selected tyrosine residues were identified with ASC structure with PDB entry 2KN6.

6. Overall Discussion

The purpose of this project was to provide a further understanding of the regulatory role of tyrosine phosphorylation of ASC in inflammasome complex formation and function. We hypothesised that tyrosine dephosphorylation plays an essential role in the regulation of ASC and the inflammasome as a whole.

The aims of this project were:

- Determine the overall effect of tyrosine phosphatase inhibition on the NLRP3 inflammasome.
- Determine the impact of tyrosine phosphatase inhibition on ASC tyrosine phosphorylation, inflammasome formation and function
- Identify the putative tyrosine residues of ASC crucial for the formation and activation of the NLRP3 inflammasome.

6.1 Conclusions

In our investigation, we initially examined the effect elicited by inhibitors of specific phosphatases. We observed that OA, NSC-87877 and PTP1Bi, inhibitors of PP2A, SHP1/2 and PTP1B, respectively, did not alter the inflammasome activity in macrophages under our treatment conditions. Notably, this study could have done with positive controls to indicate inhibition of the respective phosphatases. For instance, PP2A is activated following the dephosphorylation of Y307 by PTP1B (Geraghty et al., 2013). In order to assess whether OA or PTP1Bi were actively inhibiting their targets, a western blot of the Y307 residue of PP2A would have been carried out to assess whether PP2A or PTP1B were active.

Furthermore, this portion of the study did not include validation of these results in murine cells. Previous studies have shown that pre-LPS OA treatment in murine cells rendered the NLRP3 inflammasome inactive following treatment (Martin et al., 2014, Stutz et al., 2017). These studies involved treatment with OA prior to LPS treatment thus implicating a possible effect of OA on LPS priming. However, these previous studies suggest that PP2A plays a key role in the murine inflammasome activation. Therefore, carrying out OA treatment in murine cells after LPS priming would be crucial in further understanding the PP2A mechanism of action.

We then investigated the global tyrosine phosphatase inhibition using two broad-spectrum phosphatase inhibitors, OVN and PAO. We observed that OVN suppressed nigericin-induced inflammasome activation shown by IL-1β ELISA. Furthermore, PAO potently inhibited the activation of the NLRP3, NLRC4 and AIM2 inflammasomes. We found that PAO-mediated NLRP3 inflammasome inhibition was accompanied by a suppression of global tyrosine dephosphorylation, implicating PTP inhibition as a possible mechanism. Therefore, we established that overall PAO-mediated tyrosine phosphatase inhibition hinders the inflammasome in a mechanism that acts upstream of ASC aggregation. Although previous studies showed that global PTP inhibition by OVN induced inflammasome activation (Ghonime et al., 2012, Hoyt et al., 2016), our data reveals that nigericin-induced NLRP3 inflammasome activation in THP1DMs can be regulated by both OVN and PAO-sensitive pathways/mechanisms. We suggest that these observed differences between this study and previous studies reside in the cell type, cell species and specific treatment conditions carried out. Due to these differences, this study would have benefited from comparing murine and human cells under the treatment

conditions used here. This could have provided a better understanding on the underlying mechanisms in OVN treatment and a much more comprehensive view on inflammasome regulation by PTPs across species.

We showed that PAO inhibited tyrosine dephosphorylation of ASC mediated by nigericin treatment. This was carried out by immunoprecipitation and western blot of ASC as well as phospho-tyrosine proteins, both showing that there is a reduction in ASC phosphorylation. The immunoprecipitations were carried out with two different antibodies (anti-ASC rabbit and anti-pTyr mouse) and both immunoprecipitations showed that there was a decrease in phosphorylated ASC following nigericin treatment implying that the observed differences in ASC phosphorylation across the conditions are treatment-induced rather than technically generated. As previously discussed, ASC phosphorylation by Pyk2 via the Syk/JNK pathway was required for phosphorylation of ASC at Y146 (Hara et al., 2013b, Chung et al., 2016). Therefore, we have shown that a tyrosine phosphatase may be involved in ASC regulation.

We were unable to identify tyrosine dephosphorylation sites or the tyrosine phosphatase(s) involved; this would be vital to carry out in future work. We have also identified additional tyrosine residues that are conserved between mice and humans and are required for ASC function. The identification of these residues provides a basis to identify other tyrosine kinases involved in ASC regulation.

This project has provided *a* base on which to build on the role of tyrosine phosphorylation in inflammasome complex formation and function and specifically on the regulation of the adaptor protein, ASC.

The NLRP3 inflammasome is implicated in a host of diseases and disorders which include (but not limited to) obesity, fibrosis, cancer, Alzheimer's and autoinflammatory disorders (Aksentijevich et al., 2007, Neven et al., 2008). Understanding the complex role of tyrosine phosphorylation of ASC is necessary for the development of therapeutic interventions in these various diseases. For instance, targeting the ASC-targeting kinases and/or phosphatases disrupts the formation of an aberrant inflammasome complex and could be a valuable approach in the treatment of autoimmune disorders, including the cryopyrinopathies associated with gain-of-function mutations in the NLRP3 gene (Aksentijevich et al., 2007, Neven et al., 2008), to limit the excessive pro-inflammatory IL-1β release. Furthermore, since it has been shown that IL-1β release is dependent on caspase-1 mediated GSDMD cleavage ((Liu et al., 2016), targeting ASC regulation by phosphorylation could limit caspase-1 activation and thus prevent GSDMD-mediated membrane permeabilization and subsequent release of IL-1β and cell death.

6.2 Future Work

As we have shown that PAO inhibits PTPs and ASC tyrosine dephosphorylation upon NLRP3 inflammasome activation, it would be vital to identify which PTPs are involved and are inhibited by PAO. Therefore, with the aid of mass spectrometry, candidate PTPs can be identified. Immunoprecipitation of ASC using antibodies selective for ASC; PTPs that co-immunoprecipitate with ASC following NLRP3 inflammasome activation in the presence and absence of PAO would then be identified by protein mass spectrometry. Secondly, ASC immunoprecipitation followed by phospho-residue mass spectrometry analysis of ASC. This would aid in the identification of the tyrosine residues that are dephosphorylated upon NLRP3 inflammasome activation. Following identification of these residues, affinity ligands can be generated that possess a constitutively active phosphotyrosine at the site of the identified residue(s) to allow pull-down of candidate tyrosine phosphatases, which will also be identified by protein mass spectrometry.

It would be vital to determine the relevancy of PAO-mediated inhibition of the NLRP3, NLRC4 and AIM2 inflammasomes by assessing its effect *in vivo*. By using the peritonitis mouse model as intended in this investigation, valuable data on whether the effect seen *in vitro* is relevant can be attained. This will ultimately expand our current understanding of inflammasome regulation in a mammalian organism.

Although we have identified novel putative phosphorylation sites on ASC (Y60 and Y137), we were unable to characterise the mutants or to further identify the

candidate kinases due to time constraints. Therefore, more work involving the generation of phospho-specific antibodies and kinase inhibitor library screening, would aid in identifying the kinases involved.

In addition, the identification of putative tyrosine residues on NLRP3 would be vital. NLRP3 is implicated in a host of diseases/disorders (Tan et al., 2013, Ito et al., 2015) and as the sensor protein, the direct inhibition of NLRP3 would be important in treatment of the various diseases. Targeted mutagenesis of conserved and phosphorylation predicted tyrosine residues could be essential in understanding the regulation of the NLRP3 protein. NLRP3 is a large protein that potentially has numerous phospho-targeted tyrosines. While some studies have already shown regulation by tyrosine kinases and phosphatases (Spalinger et al., 2016, Ito et al., 2015), there still remain much to be explored about this large receptor protein.

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8. Appendix 1: Gating Strategy of Peritoneal lavage cells

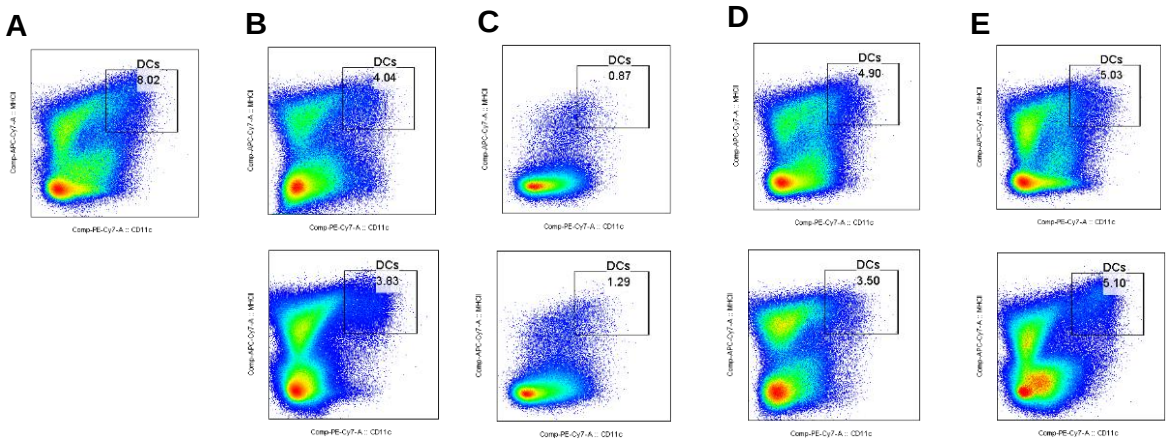

Figure 8-1: Gating strategy for dendritic cells in DMSO and ethanol with PAO as activators of peritonitis test

*Peritoneal lavage exudate cells from mice treated with PBS only(**A**), DMSO only (**B**), PAO in DMSO (**C**), ethanol only (**D**) and PAO in ethanol (**E**), were incubated for 30 minutes at 4°C with the following antibody cocktail: anti-mouse CD11b PerCP-Cy5, anti-mouse, CD11c PE-Cy7, anti-mouse F4/80 Biotin, anti-mouse Ly6C FITC, anti-mouse Ly6G PE and anti-mouse MHCII APC-Cy7. This was followed by staining with streptavidin-APC for 30 minutes at 4°C and then DAPI was added to the samples 5 minutes prior to acquisition on FACS. The cells were washed and analysed on LSR II Flow cytometer (BD). Dendritic cells (DCs) were gated as follows: live $CD11c^{+}$ $MHCII^{high}$.*

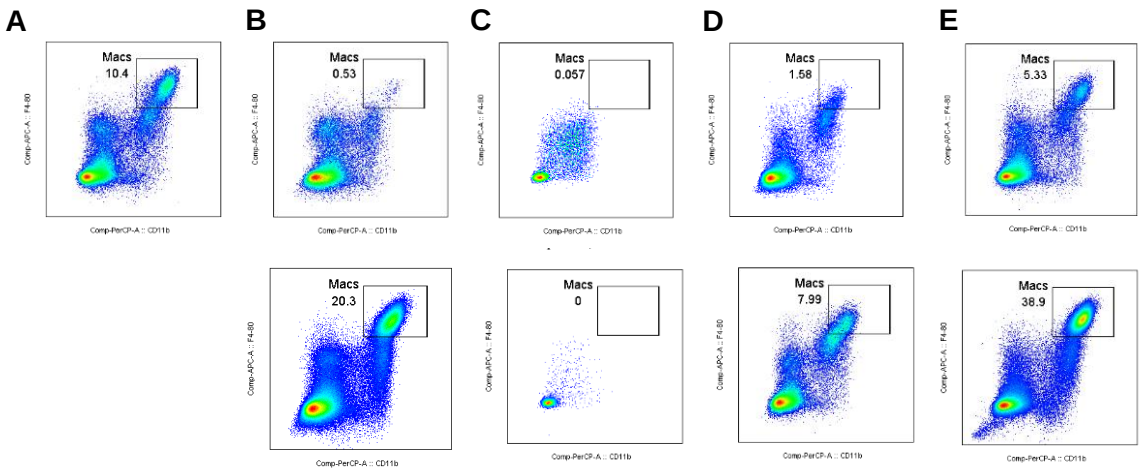

Figure 8-2: Gating strategy for macrophages in DMSO and ethanol with PAO as activators of peritonitis test

*Peritoneal lavage exudate cells from mice treated with PBS only(**A**), DMSO only (**B**), PAO in DMSO (**C**), ethanol only (**D**) and PAO in ethanol (**E**), were incubated for 30 minutes at 4°C with the following antibody cocktail: anti-mouse CD11b PerCP-Cy5, anti-mouse, CD11c PE-Cy7, anti-mouse F4/80 Biotin, anti-mouse Ly6C FITC, anti-mouse Ly6G PE and anti-mouse MHCII APC-Cy7. This was followed by staining with streptavidin-APC for 30 minutes at 4°C and then DAPI was added to the samples 5 minutes prior to acquisition on FACS. The cells were washed and analysed on LSR II Flow cytometer (BD).* Macrophages (Macs*) were gated as follows*: live $CD11b^{+}F4/80^{+}$.

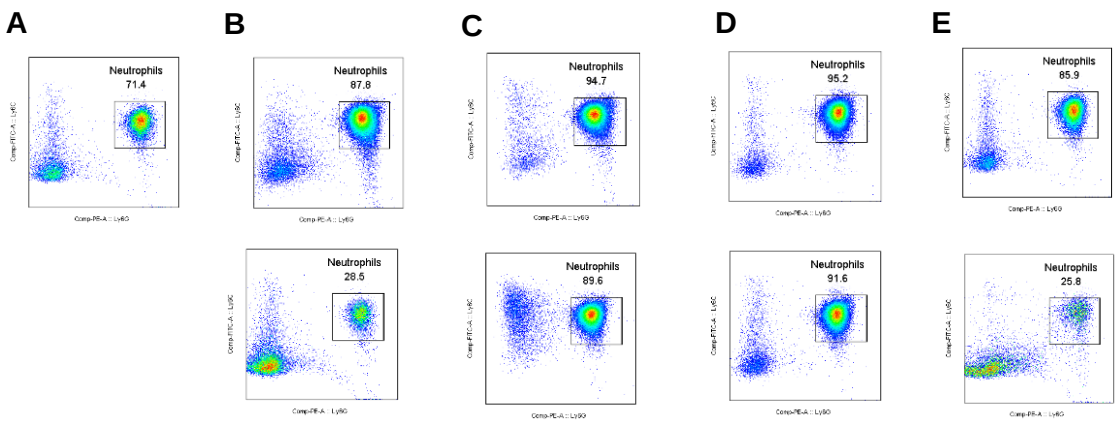

Figure 8-3: Gating strategy for monocytes in DMSO and ethanol with PAO as activators of peritonitis test

*Peritoneal lavage exudate cells from mice treated with PBS only(**A**), DMSO only (**B**), PAO in DMSO (**C**), ethanol only (**D**) and PAO in ethanol (**E**), were incubated for 30 minutes at 4°C with the following antibody cocktail: anti-mouse CD11b PerCP-Cy5, anti-mouse, CD11c PE-Cy7, anti-mouse F4/80 Biotin, anti-mouse Ly6C FITC, anti-mouse Ly6G PE and anti-mouse MHCII APC-Cy7. This was followed by staining with streptavidin-APC for 30 minutes at 4°C and then DAPI was added to the samples 5 minutes prior to acquisition on FACS. The cells were washed and analysed on LSR II Flow cytometer (BD).* Monocytes *were gated as follows*: $CD11b^{+}F4/80^{-}Ly6G^{-}Ly6C^{high}$.

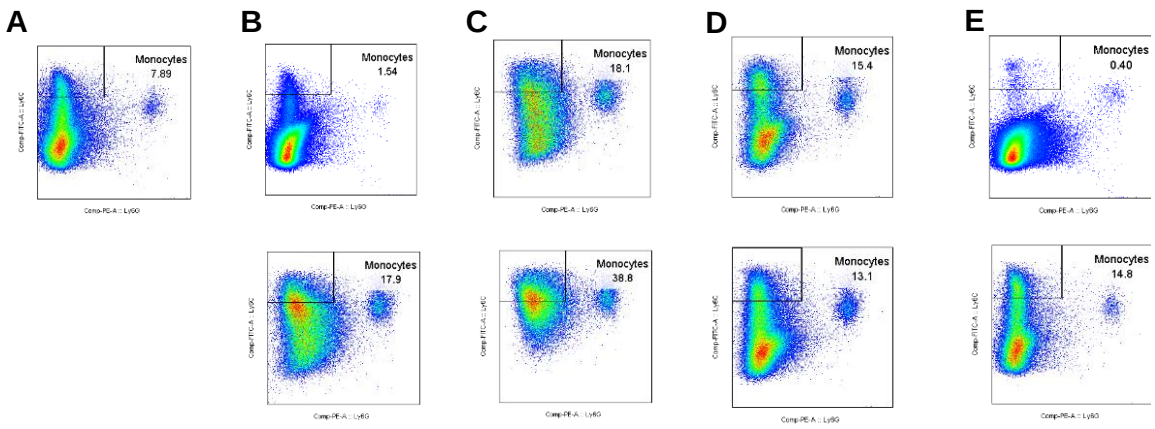

Figure 8-4: Gating strategy for neutrophils in DMSO and ethanol with PAO as activators of peritonitis test

*Peritoneal lavage exudate cells from mice treated with PBS only(**A**), DMSO only (**B**), PAO in DMSO (**C**), ethanol only (**D**) and PAO in ethanol (**E**), were incubated for 30 minutes at 4°C with the following antibody cocktail: anti-mouse CD11b PerCP-Cy5, anti-mouse, CD11c PE-Cy7, anti-mouse F4/80 Biotin, anti-mouse Ly6C FITC, anti-mouse Ly6G PE and anti-mouse MHCII APC-Cy7. This was followed by staining with streptavidin-APC for 30 minutes at 4°C and then DAPI was added to the samples 5 minutes prior to acquisition on FACS. The cells were washed and analysed on LSR II Flow cytometer (BD).* Neutrophils *were gated as follows*: $CD11b^{+}F4/80^{-}Ly6G^{+}Ly6C^{low/int}$.

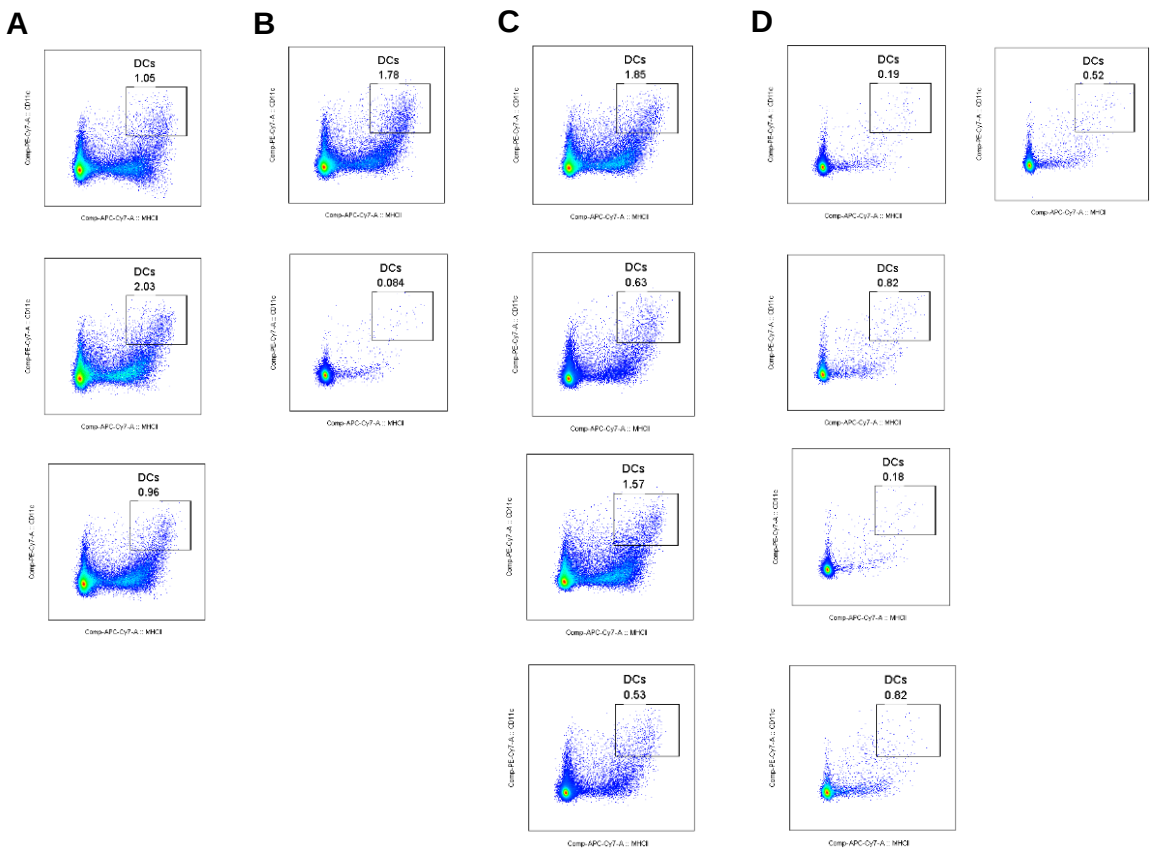

Figure 8-5: Gating strategy for dendritic cells in PAO treatment of the MSU-induced peritonitis mouse model

*Peritoneal lavage exudate cells from mice treated with ethanol only (**A**), PAO in ethanol only (**B**), MSU only (**C**) and PAO + MSU (**D**) were incubated for 30 minutes at 4°C with the following antibody cocktail: anti-mouse CD11b PerCP-Cy5, anti-mouse, CD11c PE-Cy7, anti-mouse F4/80 Biotin, anti-mouse Ly6C FITC, anti-mouse Ly6G PE and anti-mouse MHCII APC-Cy7. This was followed by staining with streptavidin-APC for 30 minutes at 4°C and then DAPI was added to the samples 5 minutes prior to acquisition on FACS. The cells were washed and analysed on LSR II Flow cytometer (BD). Dendritic cells (DCs) were gated as follows: live CD11c^{+} MHCII^{high}.*

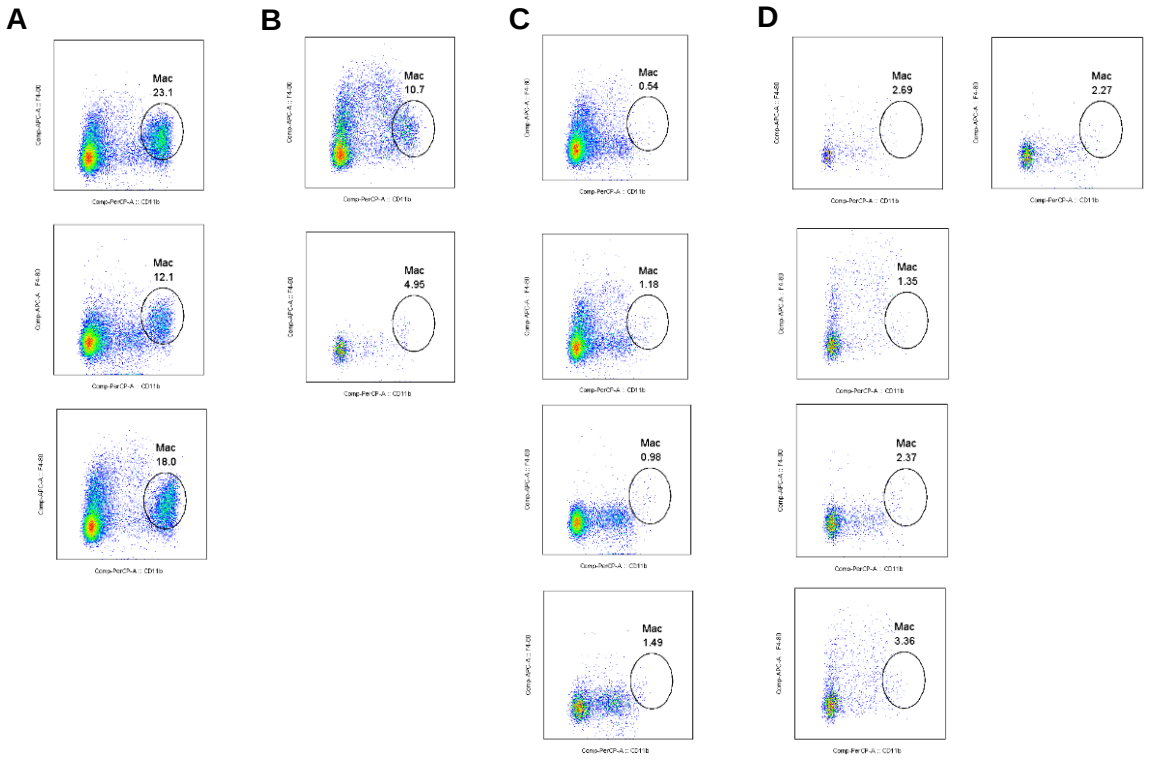

Figure 8-6: Gating strategy for macrophages in PAO treatment of the MSU-induced peritonitis mouse model

Peritoneal lavage exudate cells from mice treated with ethanol *only (**A**)*, PAO in ethanol *only (**B**),* MSU only *(**C**)* and PAO + MSU *(**D**) were incubated for 30 minutes at 4°C with the following antibody cocktail: anti-mouse CD11b PerCP-Cy5, anti-mouse, CD11c PE-Cy7, anti-mouse F4/80 Biotin, anti-mouse Ly6C FITC, anti-mouse Ly6G PE and anti-mouse MHCII APC-Cy7. This was followed by staining with streptavidin-APC for 30 minutes at 4°C and then DAPI was added to the samples 5 minutes prior to acquisition on FACS. The cells were washed and analysed on LSR II Flow cytometer (BD).* Macrophages (Mac*) were gated as follows*: live $CD11b^{+}F4/80^{+}$.

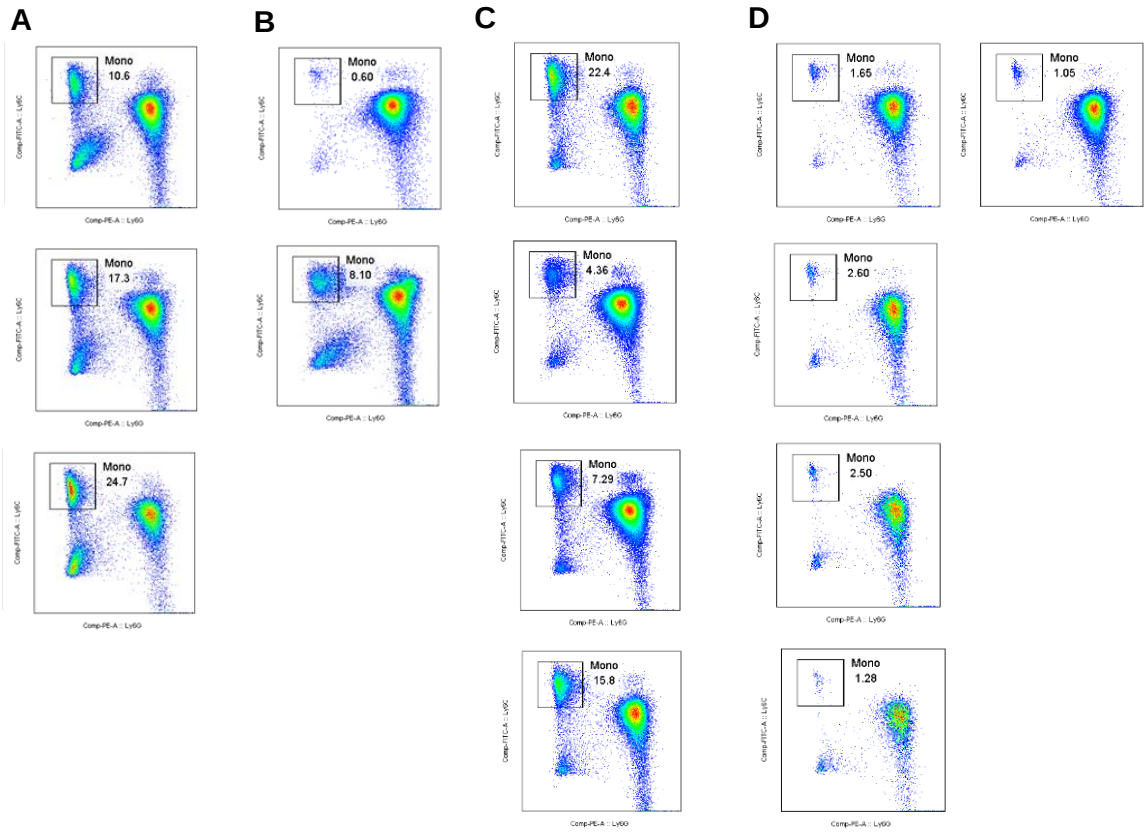

Figure 8-7: Gating strategy for monocytes in PAO treatment of the MSU-induced peritonitis mouse model

Peritoneal lavage exudate cells from mice treated with ethanol *only (**A**)*, PAO in ethanol *only (**B**)*, MSU only *(**C**)* and PAO + MSU *(**D**) were incubated for 30 minutes at 4°C with the following antibody cocktail: anti-mouse CD11b PerCP-Cy5, anti-mouse, CD11c PE-Cy7, anti-mouse F4/80 Biotin, anti-mouse Ly6C FITC, anti-mouse Ly6G PE and anti-mouse MHCII APC-Cy7. This was followed by staining with streptavidin-APC for 30 minutes at 4°C and then DAPI was added to the samples 5 minutes prior to acquisition on FACS. The cells were washed and analysed on LSR II Flow cytometer (BD).* Monocytes (Mono) *were gated as follows*: $CD11b^{+}F4/80^{-}Ly6G^{-}Ly6C^{high}$.

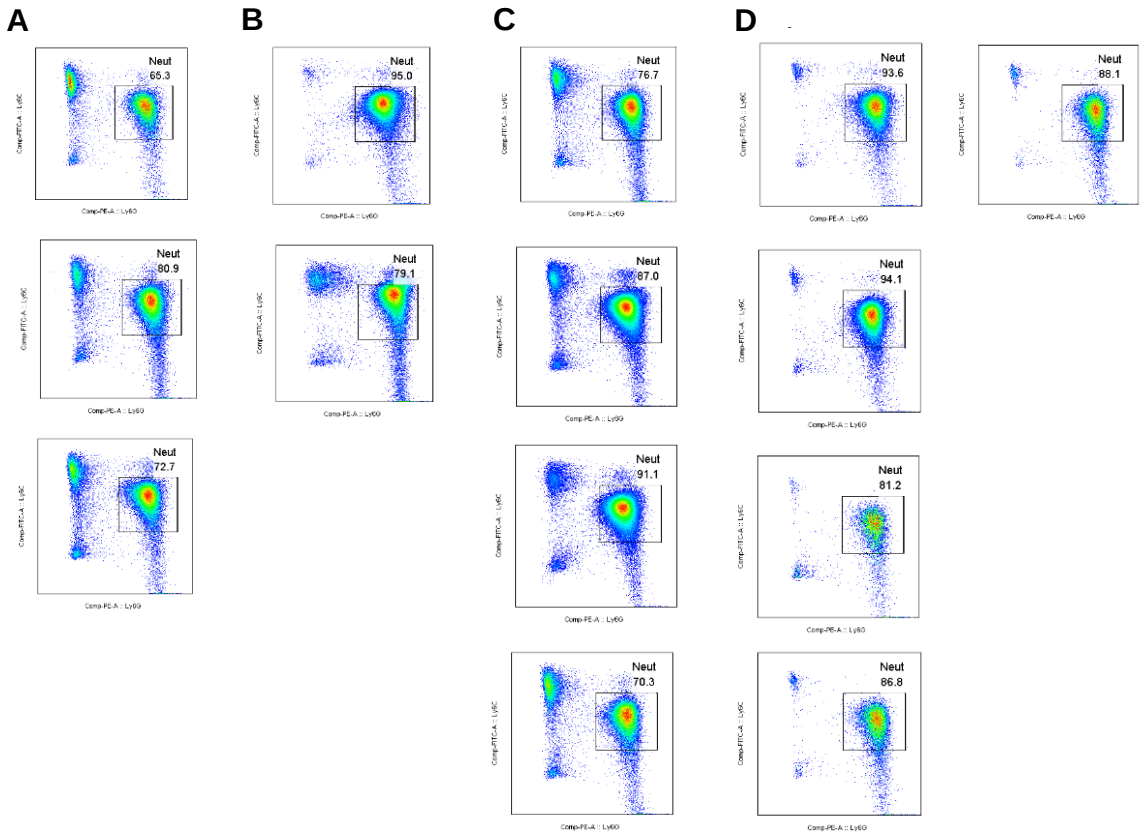

Figure 8-8: Gating strategy for neutrophils in PAO treatment of the MSU-induced peritonitis mouse model

Peritoneal lavage exudate cells from mice treated with ethanol *only (**A**)*, PAO in ethanol *only (**B**),* MSU only *(**C**)* and PAO + MSU *(**D**) were incubated for 30 minutes at 4°C with the following antibody cocktail: anti-mouse CD11b PerCP-Cy5, anti-mouse, CD11c PE-Cy7, anti-mouse F4/80 Biotin, anti-mouse Ly6C FITC, anti-mouse Ly6G PE and anti-mouse MHCII APC-Cy7. This was followed by staining with streptavidin-APC for 30 minutes at 4°C and then DAPI was added to the samples 5 minutes prior to acquisition on FACS. The cells were washed and analysed on LSR II Flow cytometer (BD).* Neutrophils (Neut) *were gated as follows*: $CD11b^{+}F4/80^{-}Ly6G^{+}Ly6C^{low/int}$.