REGULATION OF INFLAMMASOME AND NET FORMATION IN NEUTROPHILS BY PI3K SIGNALING

BY

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ABSTRACT

Introduction: The PI3K signalling pathway controls many physiological processes including inflammation, chemotaxis, proliferation, phagocytosis and microbicidal activities in immune cells such as macrophages and dendritic cells. However, it's role in neutrophils especially in the regulation of inflammasome activation and neutrophil extracellular traps (NET) formation is yet to be understood. Here, I investigated the role of p110δ isoform of the PI3K, which is uniquely expressed in leukocytes, in neutrophilic inflammatory responses.

Methods: Bone marrow derived neutrophils (BMDN) were isolated from both WT and P110δ^{D910A} mice. BMDN from both mice were challenged with LPS and Nigericin, the levels of IL-6 and IL-1β were determined by ELISA. In some experiments, NET formation was assessed using immunofluorescence microscopy and picogreen assay. Chemotaxis towards MIP1α and CXCL1 was assessed using a transwell assay system. BMDN from WT mice were treated with a pharmacological inhibitor of p110δ (CAL101) to validate the above experiments. Western blot was done to assess protein levels in pro-caspase1, ASC, NLRP3 (inflammasome proteins) and pro-IL1β. Also, WT and P110δ^{D910A} mice were challenged with LPS and NET production was measured.

Results: We observed significant impairment to core neutrophilic functions such as proinflammatory cytokine IL-6 production, NET formation and migration by neutrophils from p110δ^{D910A} mice compared to their WT counterparts. However, there was no significant difference in IL-1β or pro-caspase1 production by WT and p110δ^{D910A} neutrophils. The same result was observed in CAL101-treated BMDN. Sepsis induced P110δ^{D910A} mice were also impaired in their ability to produce NET when compared to their WT counterparts.

Conclusion: These findings suggest that signaling via the p110δ isoform of PI3K plays an important role in regulating neutrophil migration and NET formation but is dispensable for inflammasome response.

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DEDICATION

I dedicate this thesis to the Almighty God, who alone gave me the life and ability to carry out this wonderful project. Thank you, Lord, for being my banner and victory.

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LIST OF ABBREVIATIONS

AAV - anti-neutrophil cytoplasmic antibody-associated vasculitis

AIM2 - absent in melanoma

ALR - AIM2 like receptors

APAP - acetaminophen

Apoe - Apolipoprotein E

APRIL - B cell-activating factor

ASC - apoptosis-associated speck-like protein containing a caspase recruitment

ATP - Adenosine triphosphate

BAFF - a proliferation-inducing ligand

BCR – B cell receptor

BRCC3 - BRCA1/BRCA2-Containing Complex Subunit 3

c-Raf - RAF proto-oncogene serine/threonine-protein kinase

C/EBP- β - CCAAT/enhancer-binding protein beta

Ca^{2+} - calcium ion

CAPS - cryopyrin- associated periodic syndromes

CARD - caspase recruitment domain

CCL3 - Chemokine (C-C motif) ligand 3

CD14 - cluster of differentiation 14

CD3 - cluster of differentiation 3

CD40 - cluster of differentiation 40

CD86- cluster of differentiation 86

CIA - collagen induced arthritis

Cl^- - chloride ion

CLP - cecal ligation and puncture

COX-2 - Cyclooxygenase-2

CPG - cytosine–phosphate–guanine

CXCL12 - chemokine (C-X-C motif) ligand 12

CXCR4 - chemokine (C-X-C motif) ligand 4

DAG - diacyl glycerol

DAMPs - damage associated molecular patterns

DC - dendritic cells

DNA - Deoxyribonucleic acid

DSS-induced colitis

EAE - Experimental autoimmune encephalomyelitis

ECM - extracellular matrix

ER - endoplasmic reticulum

ERK1/2 - Extracellular regulated kinase 1/2

FasL - Fas ligand

FcγRs - Fc gamma receptor

fMLF - formyl-methionyl-leucyl-phenylalanine

G-CSF - granulocyte colony stimulating factor

GBP5 - guanylate binding protein 5

GLUT4 - Glucose transporter type 4

GMP - granulocyte monocyte progenitor

GPCRs - G-protein-coupled receptors

GROα - growth-regulated oncogene-alpha

H_2O_2 - hydrogen peroxide

HIV - human immunodeficiency virus

HOCl - hypochlorous acid

HPA - hypothalamic-pituitary-adrenal axis

HSC - Haematopoietic stem cells

Hsp90 - Heat shock protein 90

IAPP – a hormone released with insulin

ICAM - intercellular adhesion molecules

IFNγ - interferon gamma

IgE - Immunoglobulin E

IgG - Immunoglobulin G

IgM - Immunoglobulin M

IkBα - Inhibitor of kappa B-alpha

IKK – IKB kinase

IL-1α - Interleukin-1 alpha

IL-12 – Interleukin 12

IL-15- Interleukin 15

IL-17 – Interleukin 17

IL-18 – Interleukin 18

IL-1β – Interleukin-1 beta

IL-6 – Interleukin 6

IL-8 – Interleukin 8

iNOS - nitric oxide synthase

IRAK-1 - IL-1 receptor associated kinase 1

IRAK4 - IL-1 receptor associated kinase 4

JAK – Janus kinase

JNK – Jun N-terminal kinase

K^+ - potassium ion

LMPP - lymphoid multipotent progenitor

LPS - lipopolysaccharide

MAPK - mitogen-activated protein kinases

MARK4 - Microtubule-affinity regulating kinase 4

MCP-1 – monocyte chemoattractant protein – 1

MEK – Mitogen-activated protein kinase kinase

mg – Microgram

MHC II – major histocompatibility complex – 1

MIF - Macrophage migration inhibitory factor

MIP1α - macrophage inflammatory protein – 1

MKP-1 – Mitogen-activated protein kinase phosphatase 1

ml - Millilitre

mM – Millimolar

MNS - Methylenedioxy-β-nitrostyrene

MPO - myeloperoxidase

MPP - multipotent progenitor

MS - multiple sclerosis

MSU - monosodium urate crystals

mtDNA – mitochondria Deoxyribonucleic acid

mTOR - mechanistic target of rapamycin

MyD88 – myeloid differentiation primary response 88

Na^+ - sodium ion

NADPH - nicotinamide adenine dinucleotide phosphate

NAIP - NLR-family apoptosis inhibitory protein

NEK7 – NIMA-related kinase 7

NET - neutrophil extracellular traps

NFκB - nuclear factor kappa-light-chain-enhancer of activated B cells

ng – Nanogram

NK - natural killer

NKT - natural killer T cells

NLR - NOD like receptors

NLRC4 - NLR-family, caspase activation and recruitment domain (CARD)-containing 4

NLRP3 - NOD like receptors (NLR) family pyrin domain (PYD) - containing 3

nM – Nanomolar

NO - nitric oxide

O^{2-} - superoxide

OVA - ovalbumin

p110γ – Phosphatidylinositol three kinase gamma

p110δ - Phosphatidylinositol three kinase delta

PAD4 - protein-arginine deiminase type 4

PAMPS - pathogen associated molecular patterns

PBMC - peripheral blood mononuclear cell

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

pg – Picogram

PI3K - phosphoinositide 3 kinase

PKA - protein kinase A

PKR - Protein kinase RNA-activated

Poly I:C - Polyinosinic: polycytidylic acid

PRR - pathogen recognition receptors

PTPN22 - Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)

PYD - pyrin domain

RA - rheumatoid arthritis

RNA - ribonucleic acid

ROS - Reactive oxygen species

RPMI - Roswell Park Memorial Institute Medium

S1PR1 - Sphingosine-1-phosphate receptor 1

SGT1 - suppressor of G2 allele of skp1

SLE - systemic lupus erythematosus

SOCS - Suppressor of cytokine signaling

STAT - signal transducer and activator of transcription

TCR – T cell receptor

TF - transcription factor

TGFβ - Transforming growth factor beta

Th1 - T-helper 1 cells

Th17 - T-helper 17 cells

Th2 - T-helper 2 cells

TIR - Toll/interleukin-1 receptor

TLR4 - Toll-like receptor-4

TNFα - Tumor necrosis factor alpha

TRAIL - TNF-related apoptosis-inducing ligand

TRIF - TIR-domain-containing adapter-inducing interferon-β

TRIM31 - Tripartite motif-containing protein 31

TRX – Thioredoxin

TXNIP - thioredoxin-interacting protein

USP47 - Ubiquitin Specific Peptidase 47

USP7 - Ubiquitin Specific Peptidase 7

VCAM1 - vascular cell adhesion molecule 1

VLA4 – Very late antigen 4

WHO – world health organization

WT - Wild Type

CHAPTER 1: INTRODUCTION

1.1 Inflammation

Inflammation is a normal physiological process involving the body's mechanism of restoring homeostasis following an infection, tissue insult or internal stress(1). The microcirculation reacts non-specifically by moving fluid and immune cells into the extravascular space where tissue insult has occurred(1,2). The cardinal signs of inflammation include redness, heat, pain, swelling and loss of function(2). There are three main stages of the inflammatory pathway and regulation can occur at any of these stages. Inflammation has been classified into two - acute and chronic responses; acute inflammation of which we are most familiar with(3).

1.1.1 The inflammatory pathway

Inducers. These are the signals that activate specialized sensors of the "danger". They can be exogenous or endogenous to the host. Exogenous inducers can be microbial products such as pathogen associated molecular patterns (PAMPS) and virulent factors; or non-microbial like allergens, irritants, foreign bodies or any toxic compounds(1,4). Endogenous inducers are cell, tissue, plasma or extracellular matrix (ECM) derived and they are usually signals released from malfunctioning, stressed or dead cells, damaged tissues, endogenous crystals or products of ECM breakdown(1,5).

Sensors. Are usually pathogen recognition receptors (PRRs), plasma proteins and antibodies that are capable of identifying inflammatory inducers(6). Inducers and sensors make up the initiation stage of inflammation.

Mediators. When inflammation is induced, many inflammatory mediators are produced to amplify the signal – this is the second stage of the inflammatory process. These mediators are either plasma protein derived or secreted from cells and they have common effects on leukocyte

recruitment and the vasculature(3). There are seven groups of inflammatory mediators. First, vasoactive amines which comprises of histamines and serotonin. They are pre-produced and stored in mast cells and platelets and released upon degranulation. They can mediate vasodilation or vasoconstriction depending on the context of inflammation(1,7). Second, vasoactive peptides such as kinin and fibrin are derived when proteolysis occur by factor XII, plasmin or thrombin and they function in vasodilation(1,7). Third, are complement proteins such as C3a, -4a, -5a which function to promote leukocyte recruitment, degranulation of mast cells and consequently, vasodilation(1,7). Fourth are lipid mediators which are phospholipid derived. These include prostaglandins and leukotrienes which cause vasodilation and bronchoconstriction, respectively thereby advancing the inflammatory response; and platelet activating factors that act to activate platelets (7,8). Fifth, inflammatory cytokines such as IL-6, IL-1, TNFα, etc. which are produced by both immune and structural cells act to boost the acute phase response and activate leukocytes and the endothelium(1,7). Sixth are chemokines produced by numerous cells to increase leukocyte migration towards affected site(1,7). Lastly, diverse proteolytic enzymes such as cathepsin, matrix metalloproteinase, can function to degrade extracellular matrix thereby playing very crucial roles in host defence, leukocyte migration and tissue repair(1,7). Most mediators can in turn induce the production of other mediators or have direct effect on cells and tissues during inflammation(1). In addition to causing vasodilation and leukocyte migration, these mediators also function in metabolic and neuroendocrine activities (9).

Effectors. Cells and tissues are the most obvious effectors of inflammation. An example of how this pathway can neatly come together can be seen when lipopolysaccharide (LPS) is sensed by Toll-like receptor-4 (TLR4). This usually led to the generation of mediators such as IL-6, IL-1, TNFα which will eventually exert its effects on leukocytes, hepatocytes, endothelium,

hypothalamus and other cells or tissues(10). Allergens can be sensed by IgE that are bound to the IgE receptors on mast cells and basophils leading to the generation of histamines that would have effect on smooth muscle and endothelial cells(11). Bacteria toxins or sodium crystals can be sensed by the NOD like receptors (NLR) family pyrin domain (PYD) - containing 3 (NLRP3) inflammasome. The inflammasome regulates the maturation of IL-1β which will have an effect on leukocytes and other cells(12). Furthermore, the release of collagen from the ECM can be sensed by factor XII which activate various cascades – Kallikrein-kinin, coagulation, fibrinolytic, and complement cascade. This results in the production of mediators such as bradykinin and complements, exerting an effect on smooth muscle cells and the endothelium(1).

1.1.2 Acute inflammation

Acute inflammation involves the influx of leukocytes and plasma to the site of tissue insult and it lasts for a short duration. Initially, tissue resident macrophages and mast cells sense tissue insult via their receptor network such as TLRs and NLRs(6). This recognition triggers the production of mediators like histamines, cytokines, chemokines, etc. whose primary role is to cause vasodilation, allow influx of leukocytes (mainly neutrophils) and activate them(1). Neutrophils on the other hand attempt to resolve the "insult" by releasing toxic contents from its granules; however, it is not so good at distinguishing between invaders and host targets, and this results in damage to host tissues sometimes(13,14).

If acute inflammation is successful, it not only eliminates the insulting agent, it also resolves the inflammatory pathway and repair wounded tissues(15,16). The resolution of inflammation is marked by a flip in lipid mediators from pro-inflammatory prostaglandins to anti-inflammatory lipoxins. Lipoxins are known to halt neutrophil recruitment and enhance monocyte recruitment to encourage tissue remodelling and clearance of dead cells(15). TGFβ produced by

macrophages, as well as resolvins and protectins which belong to another group of lipid mediators, are also involved in acute inflammation resolution and tissue repair(16,17).

1.1.3 Chronic inflammation

Chronic inflammation results when acute inflammation fails to eliminate the inflammatory agent. It is usually caused by the persistence of a foreign body, toxic agent, infection or the development of autoimmunity(18). There are also changes in histological features as well. Neutrophils are replaced with macrophages, inflammatory monocytes, T cells, and plasma cells(3,18). The action of inflammatory cells leads to tissue destruction and granuloma tissue is observed which is characterized by fibrosis and angiogenesis in an attempt to heal(1).

1.1.4 Sepsis

An example of an inflammatory dysfunction is sepsis. The term sepsis is used to define a systemic response to infection (mostly bacterial infections) characterized by persistent and dysregulated inflammation. It is mainly characterized by a cytokine storm; and if untreated, may result in organ failure and even death (19). According to the WHO, about 30 million people are affected by sepsis worldwide every year and about 6 million deaths are recorded due to sepsis. It is one of the leading causes of neonate and maternal mortality and morbidity around the world (20). Recent studies have implicated gram- negative bacteria such as Escherichia coli, Pseudomonas aeruginosa and Klebsiella species as the leading cause of sepsis (21,22). Therefore, sepsis has become one of the models used to study the dynamics of inflammation in mice. Some factors that have been associated with increased risk for sepsis include chronic illness like HIV infection and cancer and genetic variations (23) According to Wang et al., the presence of single nucleotide polymorphisms in CD14 and TLR4 genes were linked to increased risk for sepsis in the Chinese population (24).

Within one hour of onset of sepsis, six steps are taken to ensure its management. This include the administration of oxygen to maintain oxygen saturation at greater than 94%, collect blood culture to confirm infection and determine the causative agent, administer appropriate antibiotics to control causative pathogen, consider intravenous fluid resuscitation towards lactate clearance, check serial lactates and commence hourly urine output measurement(23). Further clinical management might employ the use of vasopressors to increase blood pressure and inotropes to increase cardiac output(23).

There has been a need to improve the available therapy for sepsis. Corticosteroids were one of the first therapies for sepsis. However, whether corticosteroid therapy for sepsis patients is useful is still an ongoing debate. In murine model of septic shock, both low and high doses of corticosteroid have been reported beneficial (24). In contrast, it showed no benefit when used for all patients with septic shock in a clinical study(23). In 2001, a recombinant human activated protein C called drotrecogin alfa was approved for sepsis treatment. As the first biological agent approved in the united states of America for the treatment of severe sepsis, this was a perceived breakthrough. It began showing signs of decreased death risk; however, it later resulted in greater risk of severe bleeding, as activated protein C is an anticoagulant protein (25,26). This drug was therefore of no use to patients with low death risk. Another class of drugs that have been tried are statins. Statins are inhibitors of the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) which are critical for cholesterol synthesis. Statins being effective in the treatment of cardiovascular diseases was not as effective in sepsis. Kruger et al. did not find a difference in plasma IL-6 concentrations between placebo and statin groups and only patients who used statin prior to sepsis had better outcome (27).

Numerous efforts aimed at targeting the pro-inflammatory cytokine network to manage inflammatory mediated disease such as sepsis have been largely unsuccessful. Therefore, novel treatment strategies are required, and this requires a clear understanding of the pathophysiology of inflammation, including the cells that orchestrate the process.

1.1.5 Regulation of inflammation

Inflammation can be regulated by the nervous system, steroids, post translational modifications, receptors, signalling molecules like Suppressor of cytokine signalling (SOCS), other immune cells, vagus nerve activity and external stimuli like Berenil. Several studies show that steroids can regulate inflammation. Glucocorticoids, a mediator of the hypothalamic-pituitary-adrenal (HPA) axis has been shown to regulate the expression of adhesion molecules and inflammatory mediators such as L-selectin, CD18 integrins, prostaglandins and leukotrienes on activated neutrophils(28). Post translational modifications such as deubiquitination has been shown to decrease inflammation in recent studies. Douglas et al. demonstrates that OTULIN, which is a deubiquitinase, can dismantle ubiquitin chains assembled by LUBAC (a potent ubiquitin assemble complex) and this results in decreased NFκB activation, decreased apoptosis and reduced necrosis(29). The immune system may make use of decoy receptors which are receptors without a signalling domain to mop up excess cytokines. Most cytokines have decoy receptors including IL-1RII (IL-1β), DcR1/2 (TRAIL) and DcR3 (FasL) (30). SOCS proteins inhibit TLR-NFκB signalling by binding MyD88 and other signalling motifs; inhibits JAK-STAT pathway; regulate T cell selection, maturation and differentiation; and regulate mitogen activated protein kinases(31). By so doing, SOCS proteins act as powerful inhibitors of inflammation. In addition to these various regulators, immune cells function to regulate themselves via autocrine and paracrine cytokine stimulation(16,32). In addition, Niijima et al. reported that efferent vagus

signalling to the thymus is affected by afferent vagus nerve activity in response to the administration of IL-1β or LPS in rats (33).

External molecules have been shown to regulate inflammation. One such agents is diminazene aceturate (Berenil). Generally used as a trypanolytic agent, the mechanism of Berenil has recently began to unfold(34). Some studies propose the alteration of DNA conformation by interfering with DNA topoisomerase binding, heterochromatin unfolding and the binding of kinetoplast DNA to result in akinetoplast parasites as its mechanisms of action (35–37). Recently, Arowolo et al. demonstrate that Berenil has the ability to block responses induced by histamine and exerts other anti-inflammatory effects apart from its trypanocidal activity (38). Also, Kuriakose et al. strongly affirms that Berenil suppressed IL-6, IL-12 and TNF production after LPS, CPG and Poly I:C stimulation in murine bone marrow derived macrophages without interfering with TLR expression. Their result also showed that proinflammatory cytokines were suppressed due to the suppression of NF-κB p65 activity, mitogen-activated protein kinases (p38 and JNK), STAT proteins (STAT1 and STAT3) by berenil both *in vitro* and *in vivo* (39). This suggest a potent role of Berenil in regulating inflammation; however, its role in anti-inflammatory cytokines and the inflammasome is yet to be studied.

1.2 Neutrophils

Neutrophils are polymorphonuclear leukocytes produced by the bone marrow in substantial amounts daily, about 10^{11} cells daily. Human neutrophils make up about 50-70% of all leukocytes in circulation(40). These cells are usually the first line of immunity against various bacteria, fungi and protozoa and are a major hallmark of the innate immune response. They exhibit a short lifespan of about 8-12 hours in circulation and up to 2 days in tissues(40,41). It was previously thought that neutrophils are effective only during inflammation's acute phase;

however, neutrophils have been shown to influence the immune response by communicating with other innate immune cells and adaptive immune cells in both an autocrine and paracrine manner(40).

1.2.1 Neutrophil development

Neutrophils are generated from haematopoietic stem cells in the bone marrow(42). Haematopoietic stem cells (HSC) which are self-renewable differentiates into multipotent progenitor (MPP) that do not self-renew. MPPs further differentiate into a lymphoid multipotent progenitor (LMPP) which later become a granulocyte monocyte progenitor (GMP) when exposed to the right environment(42). According to Vietinghoff and Ley 2008, granulocyte colony stimulating factor (G-CSF) is necessary for GMPs to commit to the neutrophil fate (43). During microbial challenge, C/EBP- β is the key transcription factor (TF) driven by G-CSF and GM-CSF to induce granulopoiesis. However, C/EBP-α TF is crucial for steady state neutrophil production(44). The developing neutrophil's nucleus evolves from a rounded to a lobated shape. Also, since the stromal cells of the bone marrow express CXCL12 and VCAM1 which are ligands for CXCR4 and VLA4, respectively, found on progenitor cells to restrict them to the bone marrow, mature neutrophils downregulate CXCR4 and VLA4 and upregulate CXCR2 which help them leave the bone marrow(45). In addition, matured neutrophils comprise of secretory vesicles and granules used to store antimicrobials such as myeloperoxidase (MPO), elastase, defensins, matrix metalloproteinases and cathelicidins(46).

1.2.2 Neutrophil recruitment

Neutrophil migration from bone marrow into the blood involves an interplay between receptors expressed on the neutrophils, ligands expressed on bone marrow stromal cells and other ligands expressed on other cells outside the bone marrow. Mature bone marrow neutrophils

downregulate CXCR4 while bone marrow stromal cells downregulate CXCL12 - a major ligand for CXCR4, allowing those neutrophils the freedom to leave the bone marrow as they upregulate CXCR2 that can sense CXCR2 ligands such as CXCL1, 2, 5 and 8 (in humans) on megakaryocytes and endothelial cells in the blood (47,48). The mobilization of neutrophils from the blood to the site of inflammation is known as leukocyte adhesion cascade. Blood vessel endothelial cells near the site of inflammation becomes activated and upregulate E-, and P-selectin on their surfaces which bind neutrophil's glycoprotein ligands and slow them down causing them to roll on the endothelium. Next, host chemokines induce a conformational change on neutrophils and cause them to express β2 integrins, which enable firm binding to ICAM1 and ICAM2 on inflamed endothelial cells thereby arresting the neutrophils (49,50). Neutrophils then transmigrate into peripheral tissues via endothelial cell-cell junctions where ICAM expression is highest, this of course is dictated by chemokine gradient. Once neutrophils are at the site of inflammation, they are led to complete their function by following chemokine gradients such as C5a and formyl-methionyl-leucyl-phenylalanine (fMLF) (51,52).

1.2.3 Neutrophil activation

During the transition of neutrophils from the bone marrow to the blood and then tissues; neutrophil activation is a multistep process that has already began during this journey. However, full activation is obtained by proinflammatory stimuli response at the site of inflammation. Full activation of neutrophils is characterized by phagocytic capabilities, Neutrophil extracellular traps (NET) production and the release of granular proteins(40). Proinflammatory neutrophils are identified by an upregulation of CD11b, Gr1/Ly6G and a downregulation of CXCR4(52). Therefore, CD11b and Ly6G have become the key markers for identifying mouse neutrophils by flow cytometry. Infact, neutrophils have been identified accurately using Ly6G than Gr1(53).

Neutrophils are activated when PAMPs or damage associated molecular patterns (DAMPs) interact with their PRRs like TLRs, NODs, C-type lectin receptors, complement receptors, chemokine/cytokine receptors or Fc receptors(40,52,54). Also, during the activation process, neutrophils undergo priming. Here, exposure to one stimulus (e.g. LPS, chemokine, TNF, adhesion molecules or growth factors) can increase the subsequent activation response to a second stimulus(55).

1.2.4 Neutrophil functions

Cytokine and chemokine synthesis. Neutrophils can produce several cytokines including IL-6, TNFα, IL-1β, GM-CSF, IL-17, IFNγ, BAFF, APRIL, IL-10, TGFβ; and chemokines such as CXCL8/IL-8, CCL3/MIP1α, CXCL1/GROα, CCL2/MCP-1, CCL2, CCL20 and many others(56). Cytokines and chemokines are important for recruitment of different immune cell populations to the site of injury, as well as their activation and regulation of their functions. For instance, neutrophils can facilitate macrophage recruitment and activation by producing TNFα and MIP1α, which are crucial for macrophage function(57). Neutrophils can produce IL-8 at the site of inflammation, which is a chemoattractant for the recruitment of more leukocytes(57). In addition, IL-17 derived from neutrophils have been shown in recent studies to regulate the activation of natural killer T cells (NKT), infiltration of neutrophils, production of IFNγ, inflammation and tissue injury in a mouse model of kidney ischemia-reperfusion injury(58). These observations suggest that neutrophils are a major producer and regulator of the cytokine milieu.

Phagocytosis. Phagocytosis or “cell eating” is dependent on the engagement of opsonized bodies to receptors such as C-type lectin or FcγRs(59). This allows the phagocyte to enclose the

opsonized body in a vesicle known as phagosome. Next, the phagolysosome is formed, a process where the phagosome fuses with the lysosome containing preformed granules. Killing mechanisms are then initiated by hydrolytic enzymes and NADPH oxidases contained in these granules(59). Phagocytosis in neutrophils is very rapid and this is a major advantage in immune defense against pathogens(60).

Reactive oxygen species (ROS) species generation. The activation of the NADPH oxidase leads to the generation of reactive oxygen species and this is associated with phagocytosis, pathogen killing and particle binding(61). Oxygen is catalyzed into superoxide (O^{2-}) and hydrogen peroxide (H_2O_2) during respiratory burst. Hydrogen peroxide fuses with chloride ion to form hypochlorous acid (HOCl) in a reaction catalyzed by MPO enzyme(61). The killing of bacteria, fungi and other pathogens is then carried out by these oxygen derivatives. Also, upon neutrophil priming or bacterial infection, inducible nitric oxide synthase (iNOS) produces nitric oxide (NO) to complement neutrophil ROS production(62,63). A study demonstrated that mice were susceptible to spontaneous infection caused by commensal flora when lacking both iNOS and NADPH oxidase, but mice deficient in one of these was fine(64).

Degranulation. The granules found in neutrophils are antimicrobial peptides and proteinases. These granules fuses with phagosome to mediate killing. Apart from the usual MPO and elastase which are the prototypic antimicrobials, neutrophils possess many cationic antimicrobials like defensins and cathelicidins(46) that function through interacting with pathogen's negatively charged components. This leads to formation of membrane pores, inhibition of DNA/RNA synthesis via direct binding and biofilm disruption in bacteria(65). LL37, which are the best studies cathelicidin has been shown to function not only as an antimicrobial peptide but also as

an immunoregulatory agent (66). Therefore, it is evident that neutrophil antimicrobial agents have enormously contributed to host defense.

NET formation. In the last few years, NET formation has been a hot spot in neutrophil functions. According to Zychlinsky et al., neutrophils can externalize their DNA, histones and other components of its azurophilic granules such as elastase and MPO with the aim of trapping pathogens for killing(67). Although NET formation is a mechanism adopted by neutrophils for host defense, it can become detrimental to the host and cause tissue injury when not properly regulated(68). NET is fully discussed in Section 1.4.

Immunomodulation of other immune cells by neutrophils. Neutrophils can communicate with other immune cells such as dendritic cells (DC), T and B lymphocytes, natural killer (NK) cells, monocytes, macrophages and even endothelial cells. This is due to their ability to express a variety of surface molecules, receptors and cytokines that can directly or indirectly interact with other cells(13). For instance, in *in vitro* studies utilizing human neutrophils and monocyte derived DC, DC activation requires direct contact between neutrophils and DCs. In these experimental systems, DCs upregulate their MHC II, CD40, CD86 expression and IL-12 production when exposed to live neutrophils; translating to better antigen presentation and T cell response(69). In another study, neutrophils incubated with OVA peptide was able to directly present these peptides to OVA specific T cells via MHC II. Therefore, they can act as antigen presenting cells and can induce Th1 and Th17 antigen specific immune response(70). Neutrophils are also known to produce cytokines such as BAFF and APRIL required for B cell activation, proliferation and survival (71). Cytokine production in NK cells can be increased by neutrophils. During Legionella infection in mice, IL-18 derived from neutrophils is required for

the production of IFNγ by NK cells(72). The interactions between macrophages and neutrophils are very critical in the inflammation process. Recruited neutrophils at inflammatory sites can attract monocytes by secreting chemokines and antimicrobial peptides and increase macrophage's antimicrobial abilities(73). ROS production by neutrophils and the interaction of neutrophil integrins with adhesion molecules of the endothelium lead to decreased endothelial barrier integrity and encourages neutrophil recruitment(74). These findings help us appreciate the function of neutrophils in modulating the entire immune system, in inflammation and infection.

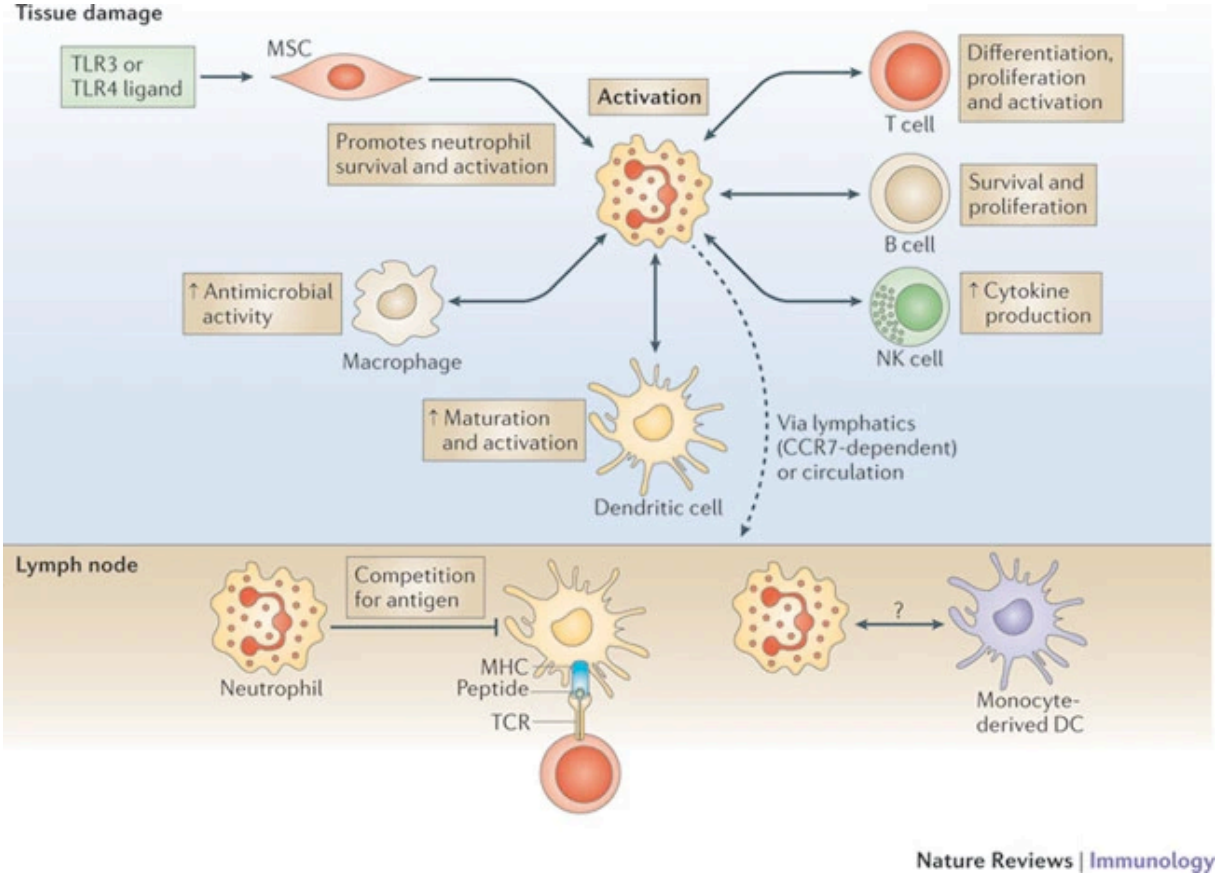


Figure 1. Neutrophil crosstalk with other cells during inflammation (13).
When tissue insult occurs, neutrophils leaves the circulation and communicate with both immune and non-immune cells to modulate their functions.

1.3 Inflammasomes

Inflammasomes are multiprotein complexes that are assembled in the cystol by intracellular PRRs when PAMPs or DAMPs are sensed(75). These intracellular PRRs, usually nucleotide-binding domain, leucine-rich repeats containing proteins (NLRs or NOD like receptors) and absent in melanoma 2-like receptors (AIM2-like receptors), once activated, form oligomers with apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC or adaptor molecule) and recruit Pro-caspase 1 resulting in the cleavage of matured caspase 1 enzyme necessary for the maturation of proinflammatory cytokines such as IL1β and IL18(75)(76). The inflammasome is also responsible for pyroptosis, an inflammatory form of cell death. Here, gasdermin D form pores in the plasma membrane and releases IL1β, IL18 and caspase 1 into the extracellular environment(76).

Inflammasomes are critical in innate immune function and have a final aim of inducing inflammation, causing pyroptosis and modulating the resulting adaptive immune response (77)(78). Basically, inflammasomes have three components – a sensor, an adaptor (mostly ASC) and an effector (mostly caspase 1). Names are given to inflammasome complexes based on their sensors or intracellular receptors and there are two major receptor classifications namely, NLR and AIM2 like receptors (ALR). Some fully recognized inflammasomes include the NLRP1, NLR-family apoptosis inhibitory protein (NAIP), NLR-family, caspase activation and recruitment domain (CARD)-containing 4 (NLRC4), AIM2 and NLRP3 (79–81). Other NLRs such as NLRP2, NLRP6, NLRP7, NLRP9, NLRP12 have shown an ability to serve as sensors and assemble the inflammasome complex but their biological function has not been well characterized and their role in inflammation and diseases are not well described (82–86).

1.3.1 NLRP3 inflammasome

The NLRP3 inflammasome is the best characterized inflammasome so far because of its range of activators. Although it have been shown to be important for defending the host against viral, bacterial and fungal infections, it has also been implicated in the pathology of many diseases (79,87–89). The NLRP3 gene encodes for a central nucleotide binding and oligomerization domain, a N-terminal pyrin domain and a c-terminal leucine rich repeat domain (90). Also, the NLRP3 does not possess a caspase recruitment domain (CARD); therefore, it requires an adaptor molecule like ASC which possesses a CARD domain to recruit pro-caspase 1 (87). Here, there is pyrin – pyrin interactions between NLRP3 and ASC to allow inflammasome formation. Myeloid cells like macrophages, neutrophils, monocytes, conventional dendritic cells express robust NLRP3 and this is negligible in lymphoid cells, plasma dendritic cells, and eosinophils. NLRP3 is usually upregulated when PAMPs such as TLRs and DAMPs are stimulated(91).

1.3.2 Activation of the NLRP3 inflammasome

The activation of the NLRP3 inflammasome has been described by a two-signal model. An interaction between PAMP and PRR on the cell surface usually provides the first signal, while the second signal comes from extracellular stress.

1.3.2.1 Signal 1: Priming the NLRP3 inflammasome

Studies have shown that NLRP3 activators alone were not sufficient to induce the inflammasome; therefore, a priming stimulant was required to activate inflammasomes in many cells including macrophages(92). Cytokine receptors, TLRs or NLRs must first be exposed to their ligands. This interaction acts to prime the cell by phosphorylating NF-κB, a transcription factor responsible for the upregulation of NLRP3 and pro-IL-1β gene activities. At resting states, NLRP3 expression is insufficient for the induction of inflammasomes(1,2). On the other hand,

ASC and procaspase-1 expression are not affected by priming. In response to ligation of TLRs, MYD88 and TRIF signalling molecules associated with NF-kB signalling pathway are responsible for inducing NLRP3 and pro-IL-1 (92).

NLRP3 induction can be dispensable for NLRP3 inflammasome activation in a fast priming process where LPS was used as a stimulant for 10 minutes(94,95). This accelerated transcription independent priming is mediated by a signalling molecule downstream of TLRs and MyD88 known as IRAK-1 (IL-1 receptor associated kinase 1)(96,97). Phosphorylation of IRAK-1 encourages inflammasome activation to occur independent of IKK signalling complex; suggesting that the role of IRAK-1 in enhancing inflammasome activation does not require NF-κB signalling(98). The priming signal is responsible for initiating NLRP3 phosphorylation mediated by JNK1 and initiate mitochondrial DNA synthesis by activating the transcription factor IRF1, both of which are essential for the activation of NLRP3(99,100). Therefore, the priming signal uses both transcriptional dependent and independent pathways to activate NLRP3 inflammasome.

1.3.2.2 Signal 2: Activating the NLRP3 inflammasome

A wide range of stimuli can activate the NLRP3 inflammasomes after priming. They include ATP, K^+ ionophores (101), pathogen associated RNA(102), bacterial and fungal components like nigericin, hyphae and other toxins (103), particulate matter(12,104,105) and heme(106). Although NLRP3 does not directly interact with these stimuli; these stimuli go on to induce many signalling events that have been shown to activate NLRP3 inflammasome like ionic flux, reactive oxygen species production, mitochondrial malfunction and lysosomal damage.

Ionic flux. Ionic flux events such as K^+ efflux, Cl^- efflux, Ca^{2+} mobilization and Na^+ influx is induced by NLRP3 stimuli and are involved in activating NLRP3 inflammasome in treated cells.

- *K^+ efflux*: This usually occur through toxin-induced plasma membrane pores or activated ATP gated P2X7 ion channel(80). In studies preceding the discovery of inflammasomes, the depletion of cytosolic K^+ has been shown to mediate the maturation and release of IL-1β from macrophages and monocytes following ATP or nigericin treatment (107). It has also been shown that K^+ efflux alone is sufficient to activate NLRP3, and the activation of NLRP3 but not AIM2 or NLRC4 inflammasome is blocked when the extracellular concentration of K^+ is high. Therefore, the most common trigger for NLRP3 inflammasomes is considered to be a decrease in intracellular K^+(108,109).
- *Na^+ influx and Cl^- efflux*: These ionic events are also involved in activating the NLRP3 inflammasomes. Blocking Na^+ influx has been shown to raise the threshold of K^+ efflux for NLRP3 activation. Also, monosodium urate crystals (MSU) have been reported to increase intracellular Na^+, promoting water influx, and this translated to decreased intracellular K^+(110). If NLRP3 inflammasome is not activated by the influx of Na^+ alone by ionophores; then, the role of Na^+ influx in activating the NLRP3 inflammasome is possibly due to its ability to regulate K^+ efflux(108). The first study to suggest the role of Cl^- efflux in inflammasome activation noted that ATP induced IL-1β maturation and secretion was enhanced when extracellular Cl^- was decreased. On the other hand, the release of IL-1β is inhibited when extracellular Cl^- was increased(111,112). Other studies suggest that Cl^- efflux can lead to ASC speck formation but not the activation of NLRP3 inflammasomes(113). This suggest that Cl^- efflux might be cooperating with other ionic events such as K^+ efflux to trigger inflammasome activation.

- *Ca^{2+} mobilization*: Although Ca^{2+} mobilization is common in many signalling pathways; its role is very controversial in NLRP3 inflammasome activation. Studies have shown that changes in intracellular Ca^{2+} can be triggered by many NLRP3 stimuli such as nigericin, ATP and particulate matter. This increase in intracellular Ca^{2+} can be mediated by phospholipase C which is activated upon NLRP3 stimulation of G-protein-coupled receptors (GPCRs), leading to Ca^{2+} efflux from the endoplasmic reticulum (ER) to the cytosol(114). Also, plasma membrane Ca^{2+} channels such as P2RX7 and lysosomes are other sources of intracellular Ca^{2+} that may trigger NLRP3 inflammasome activation(111,114,115). How intracellular Ca^{2+} can enhance the activation of NLRP3 inflammasome is still not certain; however, some studies suggest that Ca^{2+} overload of the mitochondria following increase in intracellular Ca^{2+}. This enhances mitochondrial dysfunction and leads to activation of NLRP3 inflammasome(114).

Reactive oxygen species (ROS) and mitochondrial dysfunction. Most NLRP3 stimuli can induce ROS in treated cells; therefore, ROS was thought to be the predominant signal for NLRP3 inflammasome activation with the lysosomal NADPH oxidase as the source of ROS(105,116). However, studies have shown that both pharmacological and genetic inhibition of NADPH oxidase does not affect the activation of NLRP3 inflammasomes in both human and mouse cells(104,117,118). The mitochondria have been implicated with inflammasome activation through their ability to produce ROS when dysfunctional and mitochondrial ROS has been shown to be essential for LPS & ATP-induced inflammasome activation(119). Studies have recently shown that oxidized mitochondrial DNA play a crucial role in NLRP3 inflammasome activation and mitochondria colocalizes with NLRP3 inflammasome(100,120). These results

suggest that ROS, mtDNA, and mitochondrial dysfunction play a role in NLRP3 inflammasome activation.

Lysosomal damage. NLRP3 inflammasome activation in macrophages can be induced by particulate matter such as alum, MSU, cholesterol crystals, asbestos, silica and calcium crystals(12,104,105). A critical step for activating the NLRP3 inflammasomes by particulate matter is lysosomal disruption which leads to loss of lysosomal material into the cytosol. When the lysosome is directly damaged by L-leucyl-L-leucine methyl ester (leu-leu-OMe) – a lysosomal damaging agent, inflammasome activation is triggered(104). Although the link between inflammasome activation and lysosomal damage is not so clear, some studies suggest that the acidic nature of lysosomes can result in decreased intracellular K^+ by triggering Na^+ and water influx(110). Others propose that active lysosomal enzymes such as cathepsin B are released to the cytosol after phagocytosis of particulate matter to induce NLRP3 inflammasome activation(104).

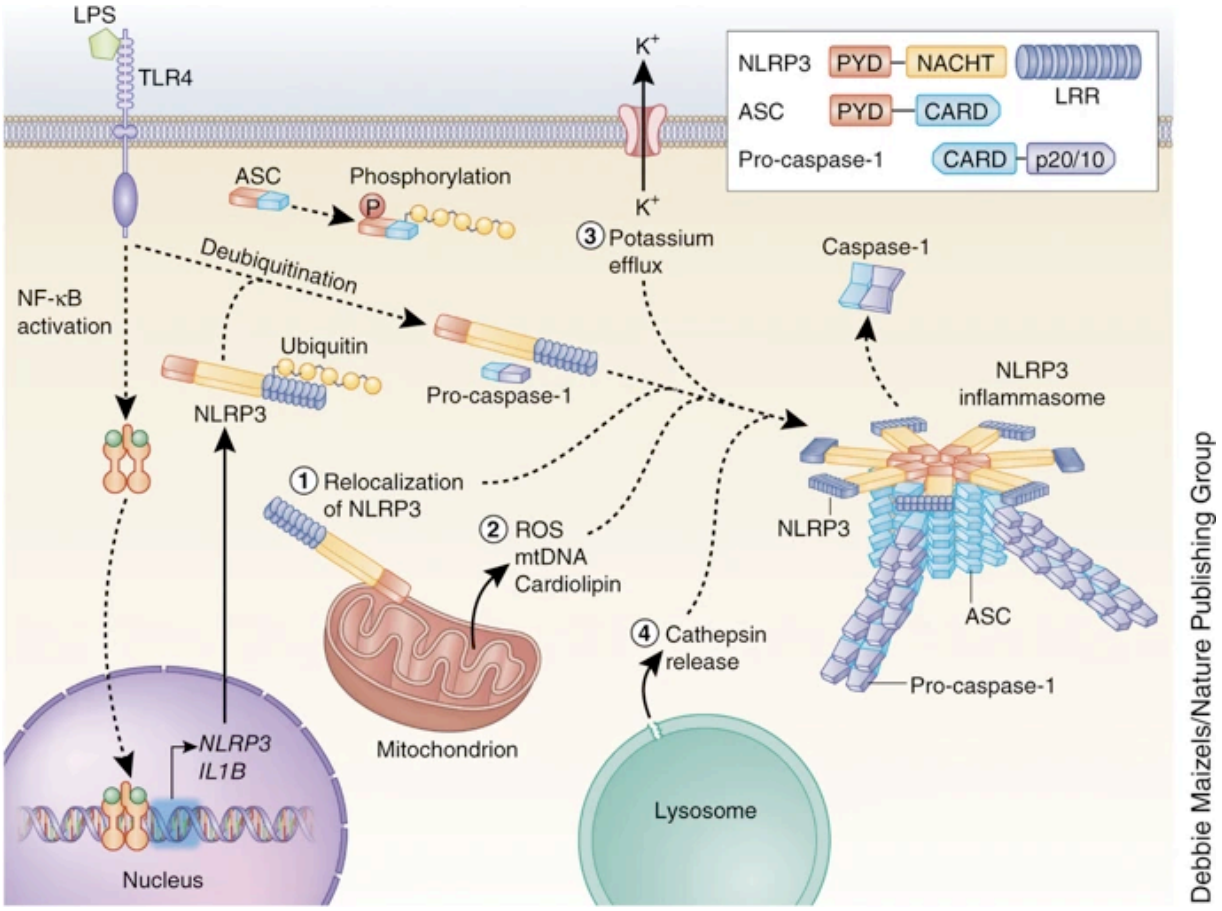


Figure 2: Mechanisms of NLRP3 inflammasome activation (75).
LPS priming induces the upregulation of NLRP3 and IL-1β through NF-κb activation. NLRP3 is licensed by deubiquitination after priming. Likewise, ASC needs to be phosphorylated and ubiquitinated linearly before inflammasome assembly can occur. Relocalization of NLRP3 to the mitochondria, release of mitochondrial products into the cytosol, potassium efflux and the release of cathepsin following lysosomal damage are the most common activating stimulus for NLRP3.

1.3.3 Regulation of NLRP3 inflammasomes

Adequate host defense is achieved with the help of inflammasomes; however, dysregulated NLRP3 inflammasome has been shown to be involved in the pathogenesis of many diseases. Hence, there is a need to properly regulate the NLRP3 inflammasome to ensure integrity of the immune system without compromising host protection. Mechanisms that have been identified to regulate NLRP3 inflammasome activation includes post translational modifications, NLRP3 interacting partners, amongst others.

Post translational modifications of NLRP3. Ubiquitination and phosphorylation are the most studied post translational modifications regulating the NLRP3 inflammasome. According to Py et al. (2013), the activation of NLRP3 inflammasomes is suppressed by a deubiquination inhibitor G5. They further established that the deubiquitination of NLRP3 during priming was carried out by BRCC36 (human)/BRCC3 (mouse) enzymes. In addition, cleavage by BRCC3 is specific to K63 linked polyubiquitin chains(121). The above research proposes that NLRP3 ubiquitination plays an inhibitory role in NLRP3 inflammasome activation. In contrast to the inhibitory role of NLRP3 ubiquitination, Pellino2, an E3 ubiquitin ligase, enhances NLRP3 inflammasome activation at some stage in the priming phase by inducing the K63-linked ubiquitination of NLRP3(122). TRIM31, a E3 ubiquitin ligase, can interact with NLRP3 and promote both K48-related ubiquitination and proteasomal degradation(123). ASC oligomerization and speck formation can also be promoted when deubiquitinases USP7 and USP47 positively regulate NLRP3 inflammasome(124). Depending on the type of ubiquitination and the ubiquitin ligase involved, NLRP3 ubiquitination can either play a positive or negative role in the regulation of the NLRP3 inflammasome.

Protein phosphorylation is a typical regulatory mechanism for many signalling pathways, the NLRP3 inflammasome inclusive. Recent studies have reported the phosphorylation of human NLRP3 at Ser295 (Ser291 in mouse) by protein kinase A (PKA) and this phosphorylation adversely regulates NLRP3 inflammasome activation by inhibiting NLRP3 ATPase activity(125). Another study by Zhang et al. suggest that NLRP3 stimuli can cause accumulation of diacyl glycerol (DAG) in the Golgi membrane leading to protein kinase D activation, subsequently phosphorylating Ser295 in human NLRP3 and promoting NLRP3 inflammasome

complex assembly(126). Also, priming signals can lead to NLRP3 phosphorylation at Ser194 mediated by JNK1, which is needed for NLRP3 activation and deubiquitination(99). Dephosphorylation of NLRP3 at Tyr861 by PTPN22, a phosphatase, has been shown to be required for NLRP3 inflammasome activation(127). Therefore, kinases and phosphatases are important for the regulation of NLRP3 inflammasomes.

Other post transcriptional modification of NLRP3 includes Nitrosylation and sumoylation of NLRP3 which can suppress its activity; ADP-ribosylation of NLRP3 can enhance NLRP3 inflammasome assembly(128–130).

NLRP3 interacting partners. Other than ASC, there are many other molecules that can interact and regulate NLRP3. Hsp90 and its co-chaperone protein SGT1 are necessary to protect NLRP3 from degradation by autophagy and proteosomes. This is achieved when Hsp90 forms a complex by recruiting SGT1 to NLPR3. This complex stabilizes NLRP3 in a state capable of signalling but inactive. Pharmacologically inhibiting Hsp90 or depleting SGT1 results in an inhibition of NLRP3 inflammasome activation(131). TXNIP, an oxidative sensor, binds TRX in resting reducing conditions; this binding is destabilized by NLRP3 inflammasome stimuli such as ATP, MSU and other uric acid crystals (due to release of ROS and its ability to oxidize TRX) resulting in TXNIP binding to NLRP3 and inflammasome activation(117). In response to ATP, nigericin and pathogenic bacteria; guanylate binding protein 5 (GBP5), a gene inducible by LPS or IFN-γ has been shown to activate NLRP3 inflammasome but not in response to particulate matter. Tetrameric GBP5 is capable of binding NLRP3's pyrin domain to enhance ASC oligomerization(132). Depletion of PKR, an RNA dependent kinase leads to reduced NLRP3 inflammasome activation and NF-κB signalling in osteoblasts(133). Macrophage migration

inhibitory factor (MIF) and Microtubule-affinity regulating kinase 4 (MARK4) are capable of interacting with NLRP3 to enhance NLRP3 inflammasome activation and spatial arrangement(134,135). NEK7 which has been involved in mitosis progression, response to DNA damage and embryo development has shown a critical role in regulating NLRP3 inflammasomes but not NLRC4 or AIM2. NEK7 uses its catalytic domain to interact with NLRP3 at its NOD and LRR domains; leading to enhanced ASC speck formation, caspase-1 activation and NLRP3 oligomerization(136,137). During NLRP3 inflammasome activation, CARD8 interacts with NLRP3 leading to an inhibition of IL-1β secretion. In diseases such as cryopytin – associated periodic syndrome (CAPS), NLRP3 is mutated and disrupts CARD8 binding, preventing the regulation of NLRP3 in the pathogenesis of CAPS(138). Overall, other molecules can interact with NLRP3 to positively or negatively regulate its inflammasome activation.

1.3.4 Cytokines produced by the inflammasome and their functions.

The assembly of inflammasomes leads to the cleavage of two major proinflammatory cytokines - IL-1β and IL-18, which are both being members of the IL-1 cytokine family. These cytokines have been implicated in numerous functions in immunity and inflammation. IL-1β and IL-18 are able to bind IL-1R1 and recruit co-receptor IL-1RAcP to induce signal transduction. MYD88 and IRAK4 are two important signalling proteins assembled by the TIR domain of IL1R1. The corresponding downstream signalling events lead to NF-κB, P38 MAPK and JNK signalling leading to IL-1β target genes induction. IL-1β and IL-18 are known to upregulate other proinflammatory genes such as IL-6, IL-8, MCP-1, COX-2, IkBα, MKP-1, IL-1α in an autocrine or paracrine manner and even act as a positive feedback to amplify IL-1β and IL-18 response(139). The cytosolic TIR domain of IL-1R1 is very similar to those found in TLRs. Hence, it is not surprising that IL-1β and IL-18 – more vigorously IL-1β - would induce the

production of several chemokines and cytokines, synthesis of nitric oxide, adhesion molecules, neutrophil chemotaxis and many other proinflammatory functions common to TLR ligands(140). The roles of IL-1β and IL-18 do not necessarily always overlap. IL-1β is known to induce fever in both mice and humans, but this property was not attributed to IL-18 when injected intraperitoneally in B6 mice(141). Secondly, IL-1β but not IL-18 has been shown to produce prostaglandin E2 by inducing cyclooxygenase-2 in human PBMCs and macrophages leading to Th17 differentiation(142). In addition, IL-1β can induce T lymphocyte proliferation via enhancing IL-2 secretion(143). On the other hand, IL-18 is commonly known as IFN-γ inducing factor. IL-18 in collaboration with IL-12 or IL-15 is necessary for IFN-γ induction and is an active participant of the Th1 paradigm. Here, IL-12 and IL-15 upregulate IL-18Rβ necessary for IL-18 signalling in T, B, NK cells and macrophages to trigger IFN-γ secretion(144). This also contributes to host protection against mycobacteria, *C. neoformans, L. major* and herpes simplex virus; as the absence of IL-18 in infected mice lead to severe infection than in the wild type mice(145–148). However, in the absence of IL-12 and IL-15, IL-18 tilts naïve T cells towards Th2 subset. IL-18 has also been shown to enhance FasL mediated killing and Perforin dependent cytotoxic activity of NK and T cells but not the expression of TRAIL(144).

1.3.5 The role of the inflammasome in diseases

The inflammasome and metabolic disorders. Inflammation has been identified as a key predictor of metabolic disorders such as obesity, type 2 diabetes mellitus and atherosclerosis. For instance, cytokine upregulation and inflammatory cytokine pathways have been associated with obesity and type 2 diabetes mellitus, while atherosclerotic plaques are characterized by immune cells accumulation(149). Amongst other cytokines, IL-1β has been implicated in the severity of metabolic disorders. Recent clinical studies suggest that a single dose of anti- IL-1β antibody

can improve secretion of insulin, suggesting a role of IL-1β in insulin signalling impairment(150)(146–149). Continuous IL-1β treatment results in decreased insulin-stimulated glucose uptake and fat formation as GLUT4 expression is reduced and glucose transport to the plasma membrane in adipocytes is impaired(151). Also, IL-1β deficiency leads to lesser atherosclerosis lesions in $Apoe^{-/-}$ mice that can spontaneously develop atherosclerosis(152). Two studies have implicated a high level of glucose and IAPP – a hormone released with insulin – in the mechanism of metabolic stress induced IL-1β. Tschopp et al. proposes the induction of ROS by glucose to directly activate NLRP3 inflammasome, while O'Neill et al. suggest that NLRP3 inflammasome activation is induced by IAPP(153,154).

The inflammasome and intestinal inflammation. Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis are linked to massive proinflammatory cytokine production including IL-1β(155). Recent studies have shown that NLRP3 signalling, ASC and Caspase-1 protect mice against DSS-induced colitis; however, their absence caused susceptibility to colitis(156). Similarly, Saleh et al. show increased intestinal inflammation, NF-κB activation and impaired tissue repair in caspase-1 deficient mice following DSS treatment(157). Trinchieri et al. hypothesized that IL-18 and not IL-1β provides protection against intestinal inflammation because IL-18 is required for intestinal homeostasis while IL-1β signalling induces other proinflammatory cytokines. True to their hypothesis, they found that mice deficient in IL-18 was susceptible to intestinal inflammation and tissue damage following DSS induced colitis(158).

The inflammasome and liver injury. NLRP3 inflammasome has been shown to amplify the immune response to acetaminophen (APAP)- induced liver injury and aggravate liver damage.

APAP causes hepatotoxicity resulting in immune cell recruitment, proinflammatory cytokine production, continuous sterile injury and dysfunctional liver. Here, mice deficient in caspase-1, ASC or NLRP3 showed reduced liver injury and reduced mortality(159).

The inflammasome and joint. The NLRP3 inflammasome is important in regulating collagen induced arthritis (CIA) in mice. This is largely because it can lead to joint destruction by mediating Th17 differentiation and IL-17 production. IL-1β inhibition has shown better results than TNFα inhibition as it can reverse established disease in addition to inhibiting disease progression(160). Increased levels of IL-18 is also present in CIA joints and blood of rheumatoid arthritis patients(161). Gout's causative crystal – uric acid- has been implicated in inflammasome activation and IL-1β secretion(12). These results suggest a role of inflammasome in joint inflammation.

The inflammasome and the skin. Inflammasomes have been shown to mediate multiple skin diseases including cryopyrin- associated periodic syndromes (CAPS) which occurs as a result of NLRP3 gene mutation. A major hallmark of CAPS is an overproduction of IL-1β by chondrocytes, monocytes and macrophages(162). IL-1β and IL-18 are also major mediators of Inflammation is a normal physiological process involving the body's mechanism of restoring homeostasis following an infection, tissue insult or internal stress(1). The microcirculation reacts non-specifically by moving fluid and immune cells into the extravascular space where tissue insult has occurred(1,2). The cardinal signs of inflammation include redness, heat, pain, swelling and loss of function(2). There are three main stages of the inflammatory pathway and regulation can occur at any of these stages. Inflammation has been classified into two - acute and chronic

responses; acute inflammation of which we are psoriasis as these patients have increased IL-1β and IL-18 in their skin lesions. Polymorphisms in CARD8, negative regulators of caspase-1 or NLRP3 genes have been shown to correlate with psoriasis susceptibility(163). Patients with atopic dermatitis have increased IL-18 serum levels and the genetic deletion or blockage of IL-18 protects atopic dermatitis mice model against dermatitis(164).

The inflammasome and the brain. The inflammasome can also mediate brain inflammations such as multiple sclerosis (MS). MS is a severe demyelination disease with Th1 and Th17 cells being the predominant mediators. Given that IL-18 and IL-1β function to activate Th1 & Th17 cells and increase vascular permeability of the blood brain vessels respectively; mice deficient in IL-1R1 does not develop EAE – the experimental model for MS(165).

The inflammasome and the lungs. Asthma patients express increased IL-1β in alveolar macrophages. IL-1β has also been implicated in lungs eosinophil infiltration, increased airway hyperresponsiveness, increased production of allergen specific IgG and IgE and increased goblet cell hyperplasia – full disease development(166). On the other hand, IL-18 confers protection in acute allergen challenge but causes lung pathology in chronic allergen challenge in mice model(167). This suggest a critical role for inflammasomes in pulmonary inflammation.

The inflammasome and cancer. The inflammasome have been implicated in several cancers due to their ability to modulate the immune system, cytokine milieu, cell apoptosis and differentiation(168). It has been reported that NLRP3 inflammasome enhances metastasis and proliferation in lung cancer as studied in human alveolar epithelial adenocarcinoma cell line. It

does this by increasing AKT, ERK1/2 and CERB phosphorylation, increasing Snail expression and decreasing E-Cadherin expression(169). Upregulation of IL-1β has been observed in breast cancer which is the number one leading cause of cancer in the world(170). Inflammasome activation, IL-1β and S1PR1 signalling in tumor infiltrating cells promote a conducive environment for breast cancer development(171). Some genetic variations in NLRP3 resulting in increased IL-1β levels have been shown to enhance susceptibility to colorectal cancer and melanoma(172).

The inflammasome and sepsis. Recent studies suggest that the inhibition of NLRP3 inflammasome could prevent or control sepsis related inflammation. Myocardial injury related to sepsis have been shown to decrease when NLRP3 is inhibited by cortistatin – an immunomodulatory factor and neuropeptide(173). Another study proposes the activation of NLRP3 in platelets of septic mice and links it to endothelial permeability, inflammation and multi organ injury observed(174). Also, the silencing of NLRP3 gene 48h before sepsis was induced, lessened bile acid concentrations, reduced cytokine levels macrophage pyrocytosis and neutrophil infiltration in hepatic tissue(175). In addition to the above studies, sepsis patients also have breathing difficulties due to the presence of elevated IL-1β and IL-18, neutrophil infiltration and lung edema in the lung tissues(176). Therefore, NLRP3 inflammasome activation can confer susceptibility to various diseases.

1.3.6 Therapeutics targeting the NLRP3 inflammasome

The inhibition of inflammasome activation has become a concern given the range of diseases it is associated with. Fortunately, the numerous signalling cascades leading to the assembly of

inflammasome can be targeted at different stages to block it. NLRP3 can be blocked directly, indirectly or blocking constituents of the NLRP3 inflammasome.

Direct inhibitors of NLRP3. One potent way to target the NLRP3 inflammasome would be to use an antagonist that can bind NLRP3 directly. MCC950, a potent and selective inhibitor of NLRP3 inflammasome directly binds to ATPase domain of NLRP3 protein to prevent ATP hydrolysis and inflammasome formation(177). *In vivo* mice studies report that MCC950 can lower pulmonary and skin inflammation and reduce EAE severity(178). Other selective inhibitors that directly bind NLRP3 and blocks it's ATPase activity include Methylenedioxy-β-nitrostyrene (MNS) which inhibits NLRP3 ATPase by modifying its cysteine residues; Tranilast, an analog of tryptophan which prevent NLRP3-ASC and NLRP3-NLRP3 interactions by binding NLRP3 NACHT domain; Oridonin, an anti-inflammatory component of *Rabdosia rubescens* – a Chinese herbal plant – can directly bind NLRP3 NACHT domain to block NLRP3-NEK7 interactions, consequently inhibiting NLRP3 inflammasome activation(179–181).

Indirect inhibitors of NLRP3. Arglabin and resveratrol have been shown to inhibit the NLRP3 inflammasome by autophagy induction and mitochondrial damage suppression in macrophages(182,183). A sulfonylurea drug used for type 2 diabetes treatment called glyburide has shown potential in inhibiting the NLRP3 inflammasome by inhibiting ATP-sensitive K^+ channels, caspase-1 activation and IL-1β release from macrophages and pancreatic β cells. It is suggested to work downstream of P2X7 but upstream NLRP3(184). JC124, a structural optimized version of glyburide has demonstrated the ability to decrease NLRP3, caspase-1, pro-IL-1β, ASC, TNFα, iNOS expression following treatment of traumatic brain injury(185). In

addition, a small synthetic molecule known as FC11A-2 can prevent the proteolytic cleavage of procaspase-1 independent of NF-κB activation(186). These drugs take advantage of the complex signalling pathways leading the NLRP3 inflammasome formation to indirectly block it.

Inhibiting constituents of the NLRP3 inflammasomes. Other molecules try to target the components of the inflammasomes. For instance, β-Hydroxybutyrate- a ketone metabolite- blocks NLRP3 inflammasome activation by inhibiting K^+ efflux and reducing ASC oligomerization(187). Parthenolide, known to exhibit anti-inflammatory properties, can inhibit the activation of caspase-1 by alkylating its cysteine residues; while Bay11-7082 blocks IKKβ kinase activity by alkylating its cysteine residue, leading to NF-κB signalling inhibition and ultimately the inhibition of NLRP3 inflammasome(188).

Targeting inflammasome products have been shown to be successful as well. Canakinumab IL-1β antibody, IL-18 binding protein, recombinant IL-1RA anakinra and decoy soluble receptor rilonacept recorded improvement in the treatment of autoinflammatory diseases such as CAPS(189).

1.4 NET formation

The formation of neutrophil extracellular traps is another strategy devised by neutrophils to limit and clear pathogens. NET formation is a term used to describe the process where neutrophils externalize their cellular contents like chromatin DNA, histones and proteins in a web-like structure (67). Although the nucleus contributes to the majority of DNA seen in NET structures, NET also consist of mitochondrial DNA(190). Neutrophils undergoing NET formation do not have the "eat me" signal on their cell surfaces; hence they are distinct from apoptotic cells.

Therefore, the remnant chromatin from NETting neutrophils are cleared by nucleases, not phagocytes(191).

1.4.1 Physiological functions of NET

The most important function of NET is to defend the host against pathogens such as bacteria, viruses and fungi(192). They are useful in immobilizing and killing pathogens due to their association with antimicrobial peptides, histones and proteases(192). Some proteins associated with NET include the serine protease – neutrophil elastase, histones, MPO, cathelicidins, defensins, calprotectin and actin(193). Another rising function of NETs is their ability to regulate inflammation. A recent study claimed that aggregate NET structures degrade cytokines and chemokines including IL-1β, IL-6, TNF-α, IL-10, MCP-1 and MIP1 due to the activity of NET-associated proteases(194). Also, neutrophil swarming might be associated with NET formation. Neutrophil swarming is a highly coordinated movement of neutrophils accompanied by neutrophil accumulation regulated by chemokines and adhesion molecules during sterile inflammation or infection. Neutrophil swarming is proposed to be amplified by NETs observed in surrounding inflamed tissue or within neutrophil clusters and this encourages immune activation(195). Furthermore, NETs are able to wall-off necrotic tissues or lumps of material with immunostimulatory activity(196). This limits the reactivity of the immune system to sterile insult. Bilyy et al. clearly show the separation of necrotic areas from surrounding healthy tissues in pancreatitis and peritonitis patients(196).

1.4.2 Mechanisms of NET formation

Different stimulants can induce different signalling leading to NET formation(197). The first described pathway of NET formation is triggered by stimuli such as PMA, microbial components, cholesterol, antibodies, mitogens and concanavalin. These stimuli in turn induce

PKC, ERK, MEK, c-Raf and AKT signalling, activating NADPH oxidase 2 (NOX2) and subsequently, neutrophil death(198).

Synergy between neutrophil elastase and MPO are necessary for chromatin decondensation at the later stages of NET formation and they do so by degrading histones(199). NE and MPO are housed in azurophilic granules and are released by NOX2-derieved ROS. However, recent studies proposed some form of NET formation can happen without NOX2 and MPO activity(197).

Histone citrullination regulated by protein-arginine deiminase type 4(PAD4) enzyme is the most common chromatin modification implicated in NET induced chromatin decondensation. PAD4 is known to convert arginine residues to citrulline, eliminating the positive charge on histones tails and weakening their binding to negatively charged DNA phosphate backbone(200). PAD4 deficient mice fails to induce histone citrullination, chromatin decondensation, NET formation, kill bacteria and are susceptible to *S. flexneri* infection(201). Inhibition of PAD4 by pharmacological inhibitors was sufficient to prevent NET formation in mouse and humans(202).

Autophagy has been implicated in NET formation in recent years. According to Remijsen et al. adequate NET formation induced by PMA requires a combination of autophagy and ROS production in human neutrophils(203). Also, neutrophils gotten from acute gout arthritic patients induce spontaneous NET release mediated by autophagy(204). Furthermore, the bacteria-derived peptide FMLP upregulated both autophagosome formation, histone citrullination and NET formation following pharmacological inhibition of the mTOR pathway(205). These studies show that autophagy is involved in regulating NET formation, although the mechanism is not yet defined.

Other NET inducers that do not require NOX2 and MPO pathway are calcium chelator A23187, a product of *streptomyces chartreusensis* which is depends on calcium flux to induce NET formation(197);and the potassium ionophore nigericin, which is a derivative of the bacteria *Streptomyces hygroscopicus.*

1.4.3 NET – double edged sword: pathology and treatment

Although NET formation plays a beneficial role in regulating the immune response to pathogens, they can also be harmful as they have been implicated in the pathogenesis of many diseases. Amongst these are sepsis, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), thrombosis and cancer.

Sepsis is characterized by multi-organ dysfunction as a result of dysregulated immune response to infection(19). Neutrophils from patients who yielded to sepsis produce less NETs invitro compared to neutrophils from patients who survived sepsis(206). This suggest a role of NET in enhancing sepsis survival in early infection; maybe, due to the antimicrobial role of NETs. Similarly, when mice were treated with DNase, the onset of sepsis was accelerated(207). In contrast, NET can damage the lungs and liver as the disease progresses by causing epithelial and endothelial cell death(208). When sepsis was induced in C57BL/6 mice by cecal ligation and puncture(CLP), the serum concentration of NETs increased significantly(209). Also, LPS-induced endotoxic shock resulted in heart, lung and liver organ damage and NET deposition in kidney tissues in C57BL/6 mice and sepsis patients(209). The administration of DNase improved survival and decreased organ failure only when DNase is given in a well-established sepsis condition(209). These results summarize the dual role of NET in early and late stages of sepsis. It provides a good knowledge of when to administer treatment.

NET formation has been implicated in autoimmune diseases. SLE, RA and anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV) patients produce autoantibodies against NET components such as citrullinated proteins(210), double-stranded DNA(211), MPO, neutrophil elastase(212) amongst many. These antibodies may develop due to the host's inability to degrade NET or as a result of continuous NET exposure(213). As shown by Yasutomo et al., genetic mutations in DNase are observed in SLE patients(214). Internalization of NET by antigen presenting cells accelerates autoimmunity in RA patients as fibroblasts containing NET enhance the production of anti-citrullinated peptide antibody(215). In SLE and AAV, glomerulonephritis is a common consequence of SLE and AAV(216). As SLE and AAV progresses, immune complexes of NET components such as MPO contribute to glomerulonephritis in human(216). A deficiency of NOX2 which is necessary for alleviating disease was observed in K/BxN mice which spontaneously induce arthritis and MRL.Fas^{lpr} mice which spontaneously develops SLE(217,218). Taken together, these results summarize the contribution of NET to autoimmunity.

Thrombosis is the clotting of blood on the wall of a blood vessel and NET has been shown to provide a scaffold and the required stimuli for thrombus formation(219). They do this by coagulating factors that are involved in blood clotting, promoting platelet and RBC adhesion to blood vessels and concentrating NET effector proteins(219–221). Citrullinated histone H3 expressing neutrophils are found on venous thrombus in mice(221). Thrombus associated neutrophils in humans are critical for deep vein thrombosis (DVT) due to their ability to form NETs that bind factor XII, the intrinsic coagulation pathway initiator(219). Heparin have been shown to remove histones from NETs leading to their disassembly, as NET formation was

decreased when DVT mice were treated with heparin(219). Therefore, NETs are not bystanders in thrombosis.

Proof also exist for a role of NET in cancer. Neutrophils gotten from tumor bearing mice possessed enhanced NET forming abilities than the WT controls. To confirm this observation, NET formation was assessed in murine pancreatic cancer and it was discovered that neutrophil autophagy enhances NET formation in pancreatic cancer(222). When DNase1 was used to degrade NET in a human pancreatic cancer cell lines; metastatic characteristics such as matrix attachment, invasion and cell migration were drastically reduced(223). Interestingly, carcinoma tumors grew slower in a PAD4 deficient mice, suggesting the role of NET formation in tumor growth(224). In addition, 4T1 breast cancer cells were colocalized with NET structure in the lungs and they have been shown to induce NETs *in vitro*. This suggest a role of NET in breast cancer metastasis(225). Overall, targeting NET formation may provide a great therapeutic option for cancer.

With the new wave of NET related research and its association in many diseases, it provides a possibility for targeting NET as therapy. In MRL/*lpr* lupus-prone mice and SLE patients, treatment with N-acetyl cysteine (a ROS scavenger) reduce NET formation, reduce autoantibodies against NET structures and also decreased severity of SLE(190,226). PF-1355, a MPO inhibitor was recently shown to decrease NET formation, neutrophil aggregation and vascular edema in a mouse model of immune complex vasculitis and this was consistent with their results in MPO deficient mice(227). PAD4 and TLR4 inhibition also display comparable effects to MPO inhibitors. Administering Cl-amidine, a PAD inhibitor, in lupus-prone MRL/*lpr* mice diminished NET formation and provided protection against skin, renal and vascular disease(228). Also, the inhibition of TLR4 with a small molecule like TAK-242 or anti-TLR4

antibody has demonstrated the ability to prevent NET formation and ROS production *in vitro* with human neutrophils(229). Another potent way to target NETs is by disrupting their architecture with DNase and promoting clearance. When DNase 1 was administered intranasally in patients with primary graft disruption and in a corresponding mice model after lung transplant, NET formation in the lung was significantly reduced, and improved oxygenation was observed(230). Recombinant DNase can also decrease autoantibody production and delay mortality in lupus-prone MRL/*lpr* mice(231).

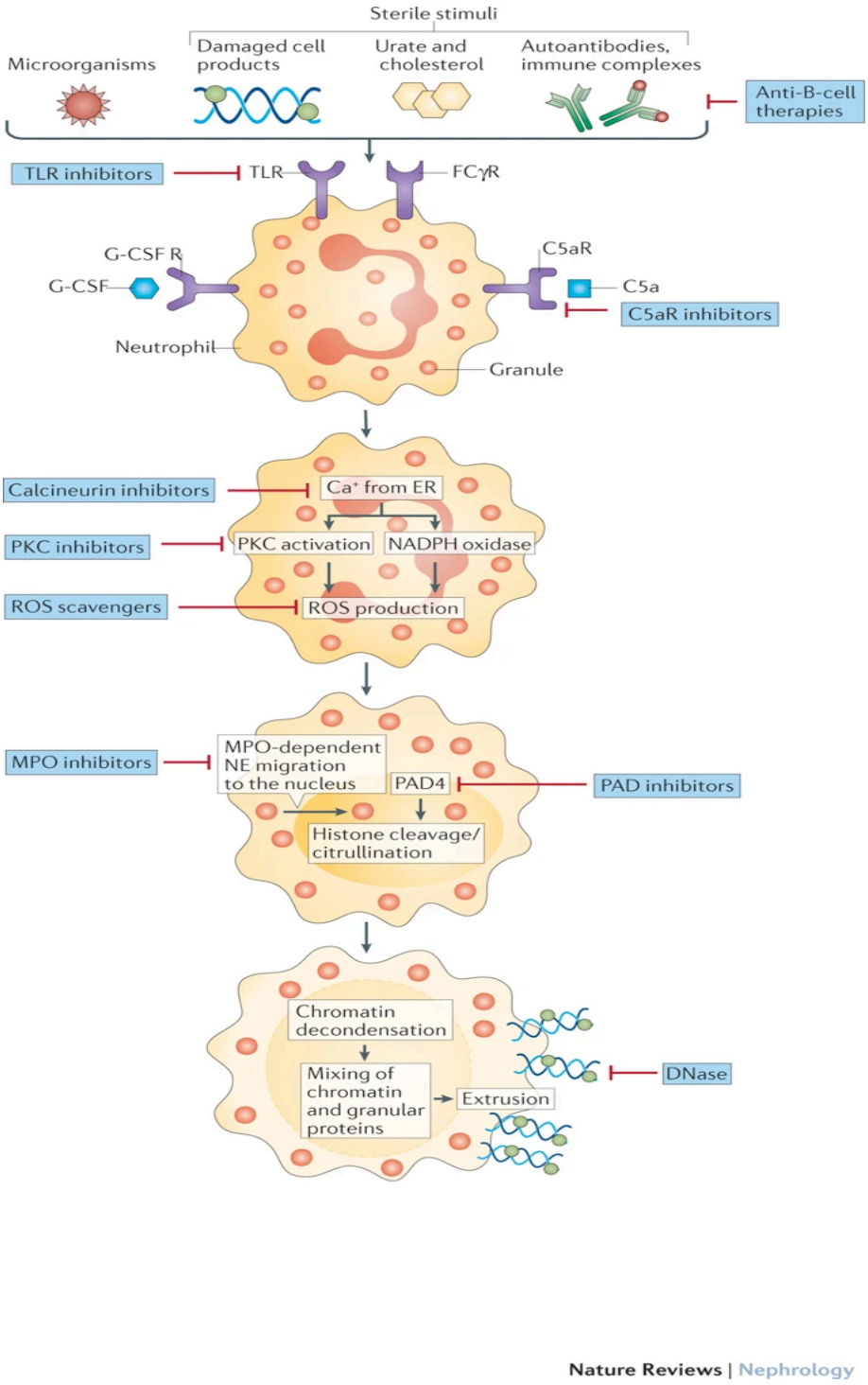


Figure 3. Potential therapeutic targets in NET formation (68).
Targeting pathways, cells, receptors and proteins that lead to NET formation such as TLR, complement proteins and their receptors, MPO, autoreactive B-cells, peptidylarginine deiminase (PAD)4, NADPH oxidase complex and/or mitochondrial activation; and the use of degradation strategies such as DNases can potentially lead to an effective treatment of diseases characterized by dysregulated NET formation.

1.5 PI3K signalling

A critical early event preceding immune cells activation that affects its overall physiologic responses including inflammation, phagocytosis, microbicidal activities and chemotaxis is the activation of the phosphoinositide 3 kinase (PI3K) pathway(232). The PI3K are family of enzymes that phosphorylate the 3'-hydroxyl group of phosphatidylinositol(233). These enzymes are associated with numerous cell functions such as cell growth, differentiation, proliferation, motility, metabolism, trafficking and survival. Numerous studies have demonstrated that PI3K signalling is involved in the activation and function of B cells, T cells, macrophages, mast cells, DCs, neutrophils and NK cells. Class IA PI3Ks are heterodimers that consist of a p85 regulatory subunit and one of the p110 catalytic subunits (p110α, p110β, p110δ). The first two isoforms (p110α and p110β) are expressed in all cells while p110δ is primarily expressed in leukocytes including neutrophils(234,235). Neutrophils possess all forms of class1A PI3K catalytic subunits(236). Unlike other catalytic subunits which are globally expressed in all cells, the p110δ is expressed in leukocytes primarily (234,235), suggesting that it may have a uunique role and/or function in the physiology of these cells. Understanding the functions of p110δ could explain how it can regulate immunity.

1.5.1 p110δ in Leukocyte signalling and activation.

Studies have shown that following BCR and TCR crosslinking with anti-IgM and anti-CD3 antibodies respectively, signalling proteins such as p-ERK, Akt and Ca^{2+} flux were decreased in B and T cells of $p110\delta^{D910A}$ mice (a knock-in mice expressing p110δ protein that is catalytically inactive)(237). In contrast, other studies have shown that TLR and NF-κB signalling are negatively regulated by p110δ activation in macrophages(238). These murine bone marrow-derived macrophages (BMDM) from $p110\delta^{D910A}$ mice also exhibit an enhanced MAPK activation(239).

Furthermore, TLR4 internalization is controlled by p110δ in dendritic cells(240). These observations suggest that p110δ plays a role in cell signalling and activation and this role can differ depending on the cell type and stimulant.

1.5.2 p110δ in Leukocyte migration.

Migration of leukocytes was thought to be originally regulated by p110γ alone due to their GPCR association (usually downstream chemokine receptors); however, p110δ signalling has also been implicated in leukocyte migration recently(241). Some studies have shown that neutrophil migration can be regulated by endothelial p110δ signalling as the selective inhibition of p110δ by IC87114 in endothelial cells but not neutrophils decreased flow cell attachment (241,242). However, the role of p110δ in bone marrow derived neutrophil as regards to migration has not been directly established. Others reported that NK cell migration to SIP and CXCL10 is regulated by p110δ and p110γ(243). B cells deficient in p110δ fail to migrate to CXCL13(241). Likewise, T cells show a reduced ability to migrate towards CCL19, CCL21 & CXCL12 chemokines and nonlymphoid antigenic tissues such as skin & gut(244). These results show the importance of p110δ signalling in migration.

1.5.3 p110δ in Leukocyte cytokine production

p110δ signalling regulates cytokine production in many immune cells. For instance, p110δ deficient NK cells are defective in their ability to produce IFN-γ, TNFα and GM-CSF cytokines following NK1.1, CD16, Ly49D and NKG2D dependent stimulation(245). Likewise, the production of IL-8 from neutrophils and IL-6 & TNFα from mast cells were impaired in p110δ deficient mice in an allergic murine model(246). The activation of AKT enhanced the production of IL-10, an anti-inflammatory cytokine, from macrophages(238). In a murine model of sepsis,

p110δ^{D910A} mice upregulated the production of IL-1β and IL-10 in the lungs simultaneously following LPS challenge(247). However, another murine sepsis study reported an increase in TNFα, IL-6, IL-1β and a decrease in IL-10 concentration in the serum following LPS challenge in p110δ deficient mice (240). From the above studies, p110δ signalling is also critical for cytokine production.

1.6 Thesis overview

1.6.1 Study rationale

With the numerous data available on the role of neutrophils in inflammation, there still exist some gaps to be filled. For example, the pathways that regulate inflammasome and NET formation in neutrophils are not completely understood. In particular, the role of PI3K signaling in orchestrating neutrophilic inflammatory responses and net formation is poorly defined. ATP-mediated ROS production has been shown to stimulate the PI3K pathway, and pharmacological inhibition of PI3K inhibits ATP-mediated caspase-1 activation in macrophages suggesting that PI3K may be involved in inflammasome activation(248). Moreover, phosphoinositide 3-kinase (PI3K) and AKT have been linked to NETosis in response to PMA and *Leishmania*(249); however, the particular catalytic subunit involved still remains unclear. Previous studies have focused mainly on the role of p110γ in NET formation, inflammation and neutrophil migration as a positive regulator and has largely neglected the role of p110δ in these processes. Hence, little is known about the role of p110δ in the context of inflammasome activation and NET formation. If PI3K signalling occurs in neutrophils, and neutrophil activity is usually associated with inflammation; then p110δ signaling like p110γ might regulate inflammation and NET formation in neutrophils.

1.6.2 Hypothesis

Signaling via the p110δ subunit of PI3K regulates inflammasome and NET formation in neutrophils.

1.6.3 Study objectives

1. Characterize neutrophils from WT and p110δ^{D910A} mice - morphology, apoptosis, migration and migratory receptors, cytokine and chemokine response.
2. Assess inflammasome response in WT and p110δ^{D910A} mice.
3. Investigate the contribution of p110δ^{D910A} signalling in NET formation.
4. Assess the effect of a pharmacological inhibitor of p110δ (CAL101) on inflammasome and NET formation in Neutrophils.

CHAPTER 2: MATERIALS AND METHODS

2.1 MICE

Female WT and P110δ^{D910A} mice on BALB/C background aged six to eight weeks were purchased from the University of Manitoba Central Animal care Services (CACS) breeding facility. The guidelines stipulated by the Canadian Council for Animal Care were followed for mice usage. The CACS provided specific-pathogen free environment, 12 hours light-dark rhythm and standard water and chow *ad libitum* for mice maintenance and survival.

2.2 BONE MARROW CELL ISOLATION

Mice were sacrificed by cervical dislocation. Lower limbs were exposed by removing fur. Hind limbs were dislocated from the hip bone, muscles and tissue were removed to expose tibia and femur. Tibia was separated from femur and ends of each bone was cut open. With a 10mL syringe, 25g needle and RPMI medium; the bone marrow was flushed until transparent. Cells were transferred to a conical tube and gently pipetted until single cell suspension was reached. Cells were centrifuged for 5 mins at 1200rpm, 1mL ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4) was used to lyse red blood cells for 5mins, washed, resuspend in 10mL RPMI and a hemocytometer was used to count cells using trypan blue dye. Cells are ready to use.

2.3 GENERATION OF MATURED MACROPHAGES FROM BONE MARROW CELLS

Bone marrow cells were resuspended at 4×10^6/ml. Conditional macrophage media was prepared by augmenting complete RPMI (RPMI supplemented with 10% heat inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) with 30% L929 supernatant. In each 15×100 mm petri dish, 1mL of 4×10^6 cells were added to 10ml conditional macrophage

media. Cells were incubated in 5% CO_2 at 37°C. 3 days after incubation, 10mL conditional macrophage media was used to top up media. Mature macrophages are ready for use after 6 days and cells are detached with the help of a cell scraper.

2.4 ISOLATION OF NEUTROPHILS FROM BONE MARROW CELLS

1× HBSS (ca^{2+}/mg^{2+} free HBSS – supplemented with 20mM Na-HEPES & 0.5% FBS) was prepared (recommended neutrophil medium). Different concentrations of percoll (GE Healthcare Life Sciences, Mississauga, Ontario, Canada) solutions (45%, 50%, 55%, 62%, 81%) were also prepared using 1× HBSS while 100% percoll was prepared by mixing 90% percoll with 10% 10× HBSS. Bone marrow cells were resuspended in 3mL of 45% percoll. In a 15mL tube, from bottom to top, different percoll densities were gently loaded: 3mL-81%, 2mL-62%, 2mL-55%, 2mL-50%, 3ml-45% percoll with cells. This was followed by a centrifuge at 2700rpm for 30mins without the brakes. Cells from 62%-81% interface were collected as mature PMN. Cells were briefly washed before use. A second way of isolating neutrophils is the use of a kit. EasySep™ Mouse neutrophil enrichment kit (19762 – STEMCELL technologies) which uses a negative selection method was also used for most neutrophil isolation steps. Percoll method of neutrophil isolation yielded ≤ 80% neutrophils while the STEMCELL kit yielded ~92% neutrophil purity from bone marrow cells.

2.5 INFLAMMASOME ACTIVATION

1μg/mL LPS was used to prime cells and incubated for 6 hours (macrophages) and 4 hours (neutrophils) at 37°C. 5μM nigericin or 5mM ATP was used as a second signal to activate the inflammasome and incubated for 45mins(macrophages) and 4 hours (neutrophils) at 37°C.

Supernatant was collected and stored for ELISA. Cells were collected and used for western blotting.

2.6 ELISA

Sandwich ELISA method was used to quantify IL-1β (ELISA max standard set mouse IL-1β; Cat# 432601 by biolegend), IL-6, TNF-α(ELISA max standard set mouse TNF-α; Cat# 430901 by biolegend), CXCL1(Mouse CXCL1/KC DuoSet ELISA; cat# DY453-05 by R&D systems), IL-12p40(ELISA max standard set mouse IL-12/IL-23 (p40); Cat# 431601 by biolegend), IL-10(ELISA max standard set mouse IL-10; Cat# 431411 by biolegend). Procedure was performed according to manufacturer's suggested protocols. Briefly, purified coating antibody was used to coat plates (50 µL/well) in an ELISA coating buffer and incubated at 4ºC overnight. Plate was washed 4-5 times in 1×PBS + 0.05% TWEEN-20. 100 µL of assay diluent (5%FBS in 1×PBS) was used to block the each well for 1 hour and washed. 50 µL of standard or sample was added/well. Standards and samples were added, and serial dilution was done in assay diluent. This was also incubated overnight at 4ºC. After washing, 50 µL of Biotin Antibody was added per well and incubated for 2hours at 37ºC. After washing, 50 µL of Avidin HRP was added per well and incubated for 40mins at 37ºC. After washing, 50 µL of ABTS peroxidase substrate or TMB substrate was added for 15mins at room temperature away from light. The reaction was stopped using 50 µL of 2N H_2SO_4 per well. After the desired color development, plate was acquired at 450nm (Spectra Max).

2.7 WESTERN BLOT EXPERIMENTS

BMDMs and BMDNs whose inflammasome were activated were collected. 200 µL of 1× laemmli sample buffer was used to directly lyse 10^6 cells - make a solution of 1× laemmli

sample buffer by mixing 2× laemmli sample buffer (Sigma Aldrich – S3401), ddH_2O and protease inhibitors (Sigma Aldrich – 11836170001). Mixture was transferred to a 1.5mL Eppendorf tube and lysed for 30 mins while vortexing every 10 mins. This was immediately followed by centrifugation for 10mins at 10,000rpm at 4 ºC. Clear lysate without cell pellet was removed to a clean Eppendorf tube and was boiled at 95ºC for 10 mins. 10µL clear lysate was loaded and resolved on SDS-PAGE polyacrylamide (4561104 – BIORAD) under denaturing conditions at 100v for 1 hour and was wet transferred to a methanol charged PVDF membrane (Amersham Pharmacia Biotech) at 100v for 1 hour. Block membrane with 5% BSA in Tris-Buffered Saline and 0.1% Tween 20 (TBST) at room temperature for 1 hour. Add primary antibody of Rabbit anti - mouse CARD8 (Abcam), Rabbit anti - mouse NLRP3, Rabbit anti - mouse Caspase1, Rabbit anti - mouse IL-1β, Rabbit anti - mouse ASC, Rabbit anti - mouse B-actin (Cell Signaling Technology, MA, U.S.A) in 2% BSA in TBST and incubate overnight in 4 ºC. Membrane was washed 4× for 5 mins with TBST the next day and secondary antibody (Goat anti – rabbit IgG – Cell Signaling Technology) was added in 2% BSA in TBST and incubate for 1 hour. Wash step was repeated and ECL substrate (RPN2232 - Amersham Pharmacia Biotech) was used to image. Membrane was stripped for 15 mins using a stripping buffer (46430 – Thermofisher Scientific) for loading controls. BIORAD image lab software on Chemidoc MP imaging system was used to measure band intensity.

2.8 FLOW CYTOMETRY EXPERIMENTS

Extracellular and intracellular flow cytometry staining used Fluorochrome - conjugated monoclonal antibodies according to the manufacturer's protocol. To summarize, FACS buffer (PBS containing 0.01% sodium azide and 5% FBS) was used to wash cells. Fc-block

(0.25µg/tube; eBiosciences) was added to cells for 10 mins on ice and washed. Fluorochrome conjugated antibodies against CD11b, Ly6G, CCR1, and CXCR2 (eBiosciences) was used to stain the surface of the cells. 2% - 4% paraformaldehyde (Sigma Aldrich) was used to fix cells after surface staining for 10 mins on ice; cells can be washed and analyzed or permeabilized with 0.1% saponin (Sigma Aldrich) in staining buffer if continuing onto intracellular staining. Predetermined concentration of fluorochrome-conjugated mAbs against MPO (Abcam) diluted in saponin buffer, was added to permeabilized cells. Azide-free Flow buffer was used for MPO staining. Annexin V and 7AAD (eBiosciences) were used to determine cell viability. BD FACS Canto II (BD Bioscience, Mississauga, ON, Canada) was used to acquire cells and Flowjo software to analyze results (Tree Star Inc, Ashland, OR).

2.9 CYTOSPIN

Isolated neutrophils (~10^5 cells) were deposited on microscope slides by cytospin centrifugation (ThermoShandon, Pittsburgh, PA). Hematoxylin and Eosin (H&E) solutions were then used to stain the slides and dried. Zeiss Primostar iLED microscope (Carl Zeiss, Ontario, Canada) was used to capture neutrophils.

2.10 NET INDUCTION

Separate poly-L-lysine coated glass coverslips (NEU-GG-12-PLL; Neuvitro) and place each one into each well of a sterile 12-well cell culture plate. Onto the center of the poly-L lysine coverslip, gently pipette about 100 µL (10^6 cells/ µL) of resuspended BMDN stimulated with 100nM PMA and/or LPS 1ug/ml (from *E. coli* [O111:B4, LPS25 – Sigma Aldrich] or *P. aeruginosa*[L9143 – Sigma Aldrich]). Cover the culture plate and incubate for 3-5 hours at 37°C in an incubator to allow to proper attachment and stimulation.

2.11 IMMUNOFLUORESCENCE MICROSCOPY

Following NET induction, cells that did not attach to poly-L-lysine coated glass coverslips were washed with PBS 3 or more times and aspirated with a pipette. 2% - 4% paraformaldehyde (Sigma Aldrich) was used to fix cells for 15 mins at room temperature. Wash PFA away and store at 4°C until ready to stain cells or continue immediately with the staining process. Ice cold methanol was used to permeabilize attached cells in a -20°C freezer for 10 mins, cells were washed with PBS and blocked immediately with 3% BSA at room temperature for 1 hour. Rabbit anti-mouse Neutrophil elastase (ab68672) and rat anti-mouse Histone H3(ab10543) primary antibodies (Abcam) were used to stain adherent cells at a dilution of 1:200 in 3% BSA at room temperature for 2 hours (or incubate overnight in a humified chamber at 4°C. Goat anti-rabbit IgG Alexa546 (A-11010 Molecular Probes) and goat anti-rat IgG FITC (CLCC40001 – cedarlane) secondary antibodies were used to stain adherent cells at a dilution of 1:1000 in 3% BSA at room temperature for 1 hours. DAPI was used to counterstain nucleus at a dilution of 1:5000 (5mg/mL) for 10 mins at room temperature. A small drop of fluorescent mounting media was used to mount coverslips unto slides, and they were visualized via a Zeiss CSU-X1M 5000 spinning Disc Confocal Microscope (Carl Zeiss, Ontario, Canada) with a 63x oil immersion objective.

2.12 RNA ISOLATION, CDNA SYNTHESIS AND RT PCR

After inflammasome activation invitro, trizol reagent was used to isolate BMDM and BMDN's RNA. About 300µl of trizol reagent was added to 10^6 cells and homogenized by pipetting immediately after treatment without washing cells. The mixture I transferred to a 1.5ml Eppendorf tube and left to lyse for 5 minutes. 0.2ml of chloroform per 1ml trizol used was added to the same Eppendorf tube and mixed. This was incubated for 2-3 minutes and centrifuged at

12000xg at 4°C for 15 minutes. The top aqueous phase containing the RNA was transferred into a new tube and 0.5ml isopropanol per 1ml trizol used was added to precipitate the RNA. This was incubated for 10 minutes and centrifuged for 10 minutes at 12000xg at 4°C. The supernatant was decarded and 1ml of 75% alcohol per 1ml of trizol used to added to wash the RNA pellet. Mixture was centrifuged for 7500xg at 4°C for 5 minutes, supernatant removed, air dried, RNA pellet solubilized in 20µl RNase-free water and heated for 10 minutes in a water bath set at 55°C. RNA can be stored at -80°C or immediately converted to cDNA. cDNA conversion was done by a first strand cDNA synthesis kit (E6300L – New England Biolabs) according to manufacturer's protocols and PCR was performed using Luna universal qPCR master mix (M3003X - New England Biolabs) also according to manufacturer's protocols.

No.	Name	Sequence
1.	Procaspase 1	F - ACAAGGCACGGGACCTATG R - TCCCAGTCAGTCCTGGAAATG
2.	ProIL1b	F - GCAACTGTTCCTGAACTCAACT R- ATCTTTTGGGGTCCGTCAACT
3.	NLRP3	F – ATTACCCGCCCGAGAAAGG R - TCGCAGCAAAGATCCACACAG
4.	ASC (PYCARD)	F – CTTGTCAGGGGATGAACTCAAAA R - GCCATACGACTCCAGATAGTAGC
5.	GAPDH	F - AGGTCGGTGTGAACGGATTTG R - TGTAGACCATGTAGTTGAGGTCA

Table 1. List of primers used in RT qPCR.

2.13 TRANSWELL MIGRATION ASSAY

BMDN were tested for their ability to migrate towards CXCL1 and MIP1α chemokines. Here, 10^6 neutrophil cells in 100µl were placed on the top chamber containing a permeable membrane with a 3µm pore size and the chemokine prepared in 500µl complete RPMI was placed in the

bottom chamber. After 1,2 and 3 hours; cells that migrated towards the chemokines were counted using a hemocytometer.

2.14 SEPSIS INDUCTION

This was done by injecting LPS from *E. coli* (O111:B4, LPS25 – Sigma Aldrich) into WT and P110δ^{D910A} mice intraperitoneally.

2.15 PICOGREEN ASSAY

Following NET induction invitro or collection of peritoneal wash & serum from LPS treated mice; Quanti-iT PicoGreen dsDNA assay kit (P11496 - Invitrogen) was used to quantify NET formation and this was done according to the manufacturer's protocol. To summarize, a working concentration of Quanti-iT PicoGreen reagent and DNA stock solution (standard) in 1x TE buffer was prepared and the standard dilutions was made according to manufacturer's instructions. 100µl of standards and samples were first added into the 96 well plate. A working concentration of 100µl Quanti-iT PicoGreen reagent was added to each well. This was mixed by light shaking and incubated for 2-5 minutes at room temperature. A synergy Neo2 multi-mode reader was used to determine the fluorescence following incubation using a monochromator-based method with an excitation wavelength of 480nm and an emission wavelength of 520nm. The data was collected from the top with 10 reads per well. Fluorescence value of blank was subtracted from those of the samples and the resulting data was used to generate a standard curve where the DNA concentration could be calculated from the resulting fluorescent values.

2.16 DIMINAZENE ACETURATE (BERENIL)

BMDN and BMDM from WT mice were treated with 10μg/ml Berenil for 4 hours before inflammasome activation to assess the function of Berenil treatment of inflammasome formation and cytokine production.

2.17 IDELALISIB (CAL101)

BMDN and BMDN from WT mice were treated with 10μm CAL101/Idelalisib which is a pharmacological inhibitor of P110δ for 4 hours prior to inflammasome activation to assess its effect on inflammasomes, NET formation and cytokine production.

2.18 STATISTICAL ANALYSIS

Most results were represented in bar graphs and were presented as the mean ± SEM. One way or two-way analysis of variance (One-way or Two-way ANOVA) was used for comparison of cytokine & chemokine production, neutrophil migration, chemokine receptor expression and inflammasome complex protein expression between the different mice groups using Prism program (GraphPad Software Inc., CA, USA). If $p \leq 0.05$, the result is considered to be significant.

2.19 ETHICAL STATEMENT

The University of Manitoba Human and Animal Ethics Review Boards approved and reviewed all animal studies that supported this work.

CHAPTER 3: RESULTS

3.1 WT and P110δ^{D910A} neutrophils have similar morphology, survival rate *in vitro*, and surface markers expression.

PI3K has been shown to regulate many cell functions including survival and signalling(234). I sought to find out whether P110δ deficiency in neutrophils would affect basic structure and functions such as survival and expression of surface markers. To address this, I isolated bone marrow-derived neutrophils (BMDN) from WT and P110δ^{D910A} mice. About 10^5 cells were immobilized to slides by cytospin and stained with Giemsa solution to characterize neutrophil morphology. Next, WT and P110δ^{D910A} BMDN were then assessed for their ability to express CD11b and Ly6G; which are two surface markers that characterize neutrophils. WT and P110δ^{D910A} BMDN were treated with LPS at different concentrations (0,100,1000) ng/mL for 48 hours and later stained with Annexin V and 7AAD to assess their ability to survive *in vitro* in LPS. I observed similar numbers and morphological structure of neutrophil in both WT and P110δ^{D910A} BMDNs (Fig. 4A). In addition, both WT and p110δ^{D910A} BMDN express the similar levels of CD11b and Ly6G molecules on their surfaces (Fig. 4B). Also, WT and P110δ^{D910A} BMDN have similar survival rates irrespective of the presence or absence of LPS or an increase in its concentration (Fig. 4C). Collectively these results show that WT and P110δ^{D910A} BMDNs do not differ morphologically and their response to LPS stimulation were identical, suggesting that any difference in inflammasome or NET formation between WT and P110δ^{D910A} BMDN would not be due to differences in any of these basic neutrophil characteristics.

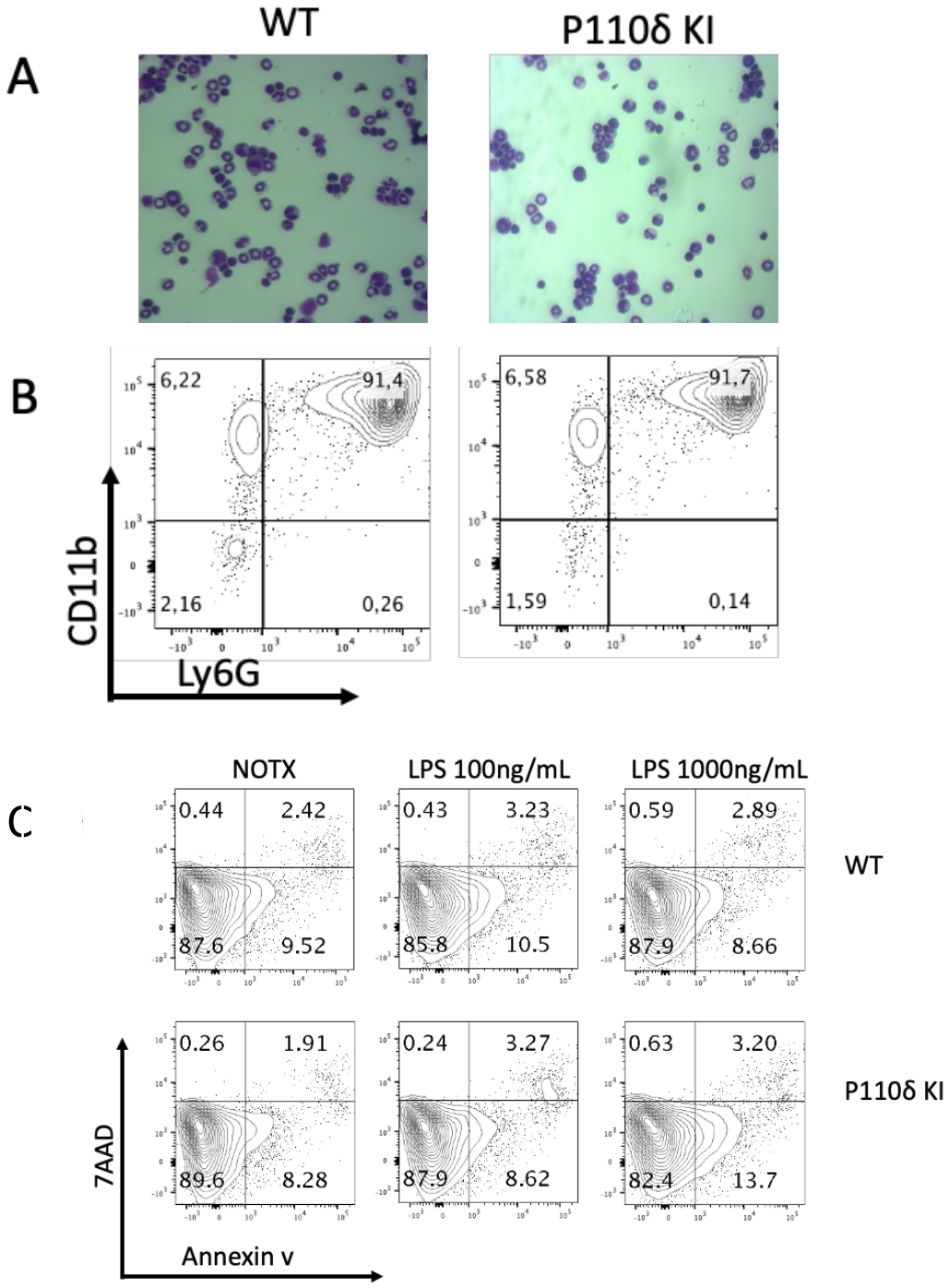


Figure 4. WT and P110δ^{D910A} neutrophils have similar morphology, survival rate *in vitro*, and surface markers expression.

Neutrophils from wild type (WT) and P110δ^{D910A} mice were isolated from bone marrow and stained with Giemsa stain to assess morphology (A). Some cells were stained with anti-Ly6G and CD11b antibodies (B), or stimulated with or without different concentrations of LPS, and stained with 7AAD and Annexin V dyes to assess cell viability (C) by flow cytometry

3.2 P110δ^{D910A} neutrophils have reduced chemokine receptor expression and impaired migration compared to WT neutrophils.

Neutrophils are one of the first responders to the site of an inflammation response in tissues. In both *in vitro* and *in vivo* models of inflammation, a reduction but not total abolition in migratory abilities was observed in neutrophils and macrophages from P110γ −/− mice (ref). This indicates that other PI3K subunits might have a role to play in cell migration(250,251). Therefore, I wanted to assess the effect of P110δ^{D910A} mutation on neutrophil migration. A transwell migration assay was used to achieve this. BMDN (top chamber) was exposed to two chemokines (CXCL1 and MIP1α) and the number of neutrophils they attracted over time was counted. I observed that P110δ deficient neutrophils were defective in their ability to migrate towards the chemokines, especially at the early time points (Fig. 5A). Next, I wanted to assess if this observed defect in migration was due to reduced expression of their corresponding chemokine receptors on P110δ^{D910A} neutrophils. To carry out this study, I exposed BMDN to LPS and stained them for expression of CXCR2 and CCR1, which are receptors for CXCL1 and MIP1α, respectively. Following flow cytometry analysis, I observed that P110δ^{D910A} neutrophils expressed less CXCR2 and CCR1 when compared to their WT counterparts even without LPS stimulation (Fig. 5B, C). Therefore, P110δ^{D910A} neutrophils have impaired migration due to their inability to sense chemokines via its chemokine receptors.

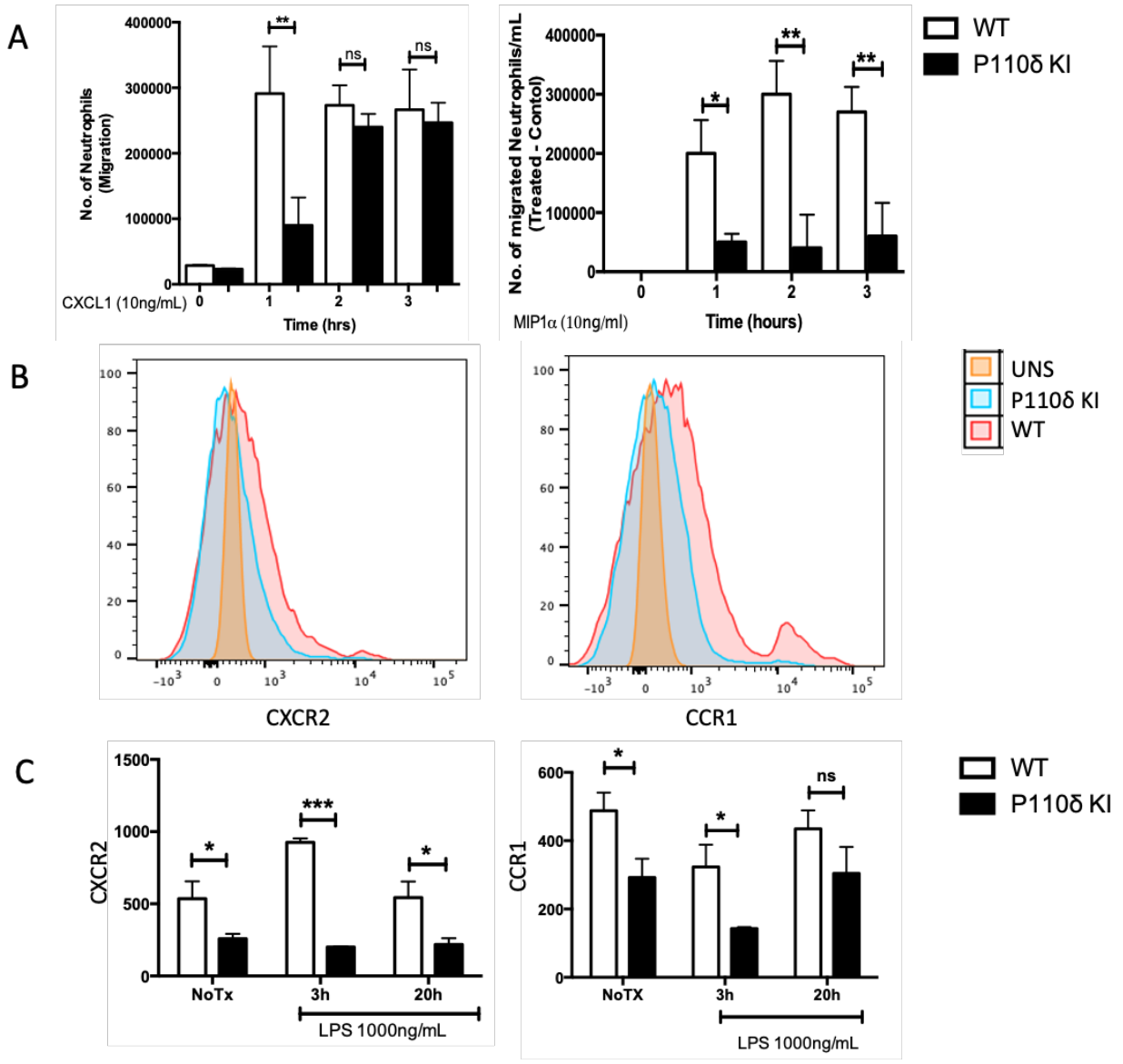


Figure 5: P110δ^{D910A} neutrophils have impaired migration and chemokine receptors compared to WT neutrophils.

Bone marrow-derived neutrophils from wild type (WT) and P110δ^{D910A} mice were assessed for migration towards CXCL1 and MIP1α (A) gradients using a transwell migration assay. The expression of CXCR2 and CCR1 receptors by neutrophils from WT and P110δ^{D910A} mice were also assessed by flow cytometry. Shown are histograms (B) and mean fluorescent intensity (MFI, C) after stimulation with or without LPS. ns = not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

3.3 CAL101 at different concentrations does not impair survival of neutrophils.

Given that we do not normally knock-in genes in humans in our attempt to regulate inflammation, using a pharmacological inhibitor of P110δ called CAL101 or Idelalisib is ideal. This would be the best way to translate my result to real situations in humans. Before that, I wanted to test CAL101 at different concentrations to make sure it does not impair the survival of neutrophils. BMDN were isolated from both WT and P110δ^{D910A} mice, treated with CAL101 at different concentrations (0,0.1,1,10) μM for 4 hours and later stained with Annexin V and 7AAD to assess their ability to survive *in vitro* in CAL101. WT and P110δ^{D910A} BMDN have similar survival rates irrespective of the presence or absence of CAL101 or an increase in its concentration (Fig. 6A).

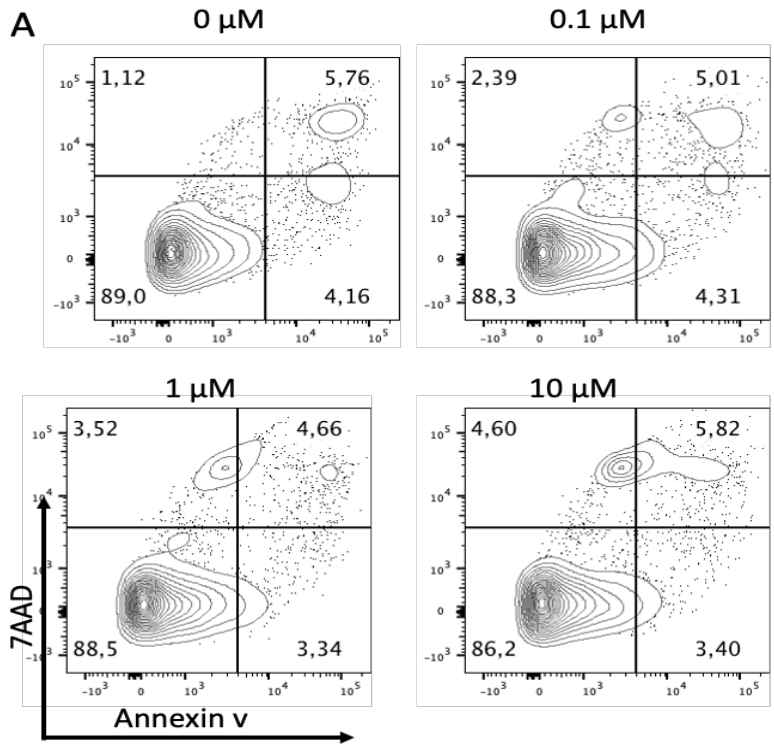


Figure 6. CAL101 at different concentrations does not impair survival of neutrophils. Neutrophils from wild type (WT) and P110δ^{D910A} mice were isolated from bone marrow, treated with CAL101 at different concentrations (0,0.1,1,10) μM for 4 hours and stained 7AAD and Annexin V dyes to assess and cell viability by flow cytometry.

3.4 P110δ^{D910A} myeloid cells have a defect in cytokine and chemokine production post LPS stimulation.

Next, I wanted to assess the implication of P110δ mutation on proinflammatory response in neutrophils. Before that, it was wise to first assess proinflammatory response in macrophages as they are easier to work with, and many studies have used macrophages to assess proinflammatory cytokine response(84,95,248). Therefore, I used these cells to optimize efficiency of my system. Bone marrow-derived macrophages (BMDMs) were isolated from both WT and P110δ^{D910A} mice and stimulated with LPS for 6 hours and the culture supernatant fluid was collected and assayed for IL-6 and TNF-α protein levels by ELISA. I observed that P110δ^{D910A} macrophages were defective in both IL-6 and TNF-α production when compared to their WT counterparts (Fig. 7A, B). Now that I know my system is working, I went on to assess the effect of P110δ mutation on cytokine and chemokine production in neutrophils. BMDNs were stimulated with LPS at different concentrations (0,10,100,1000 ng/mL) for 4 hours, and the culture supernatant fluids were assayed for IL-6, TNF-α and CXCL1 protein levels by ELISA. I noticed that BMDNs from P110δ^{D910A} produced decreased amounts of IL-6, TNF-α and CXCL1 (Fig. 7C-E) compared to their WT counterparts.

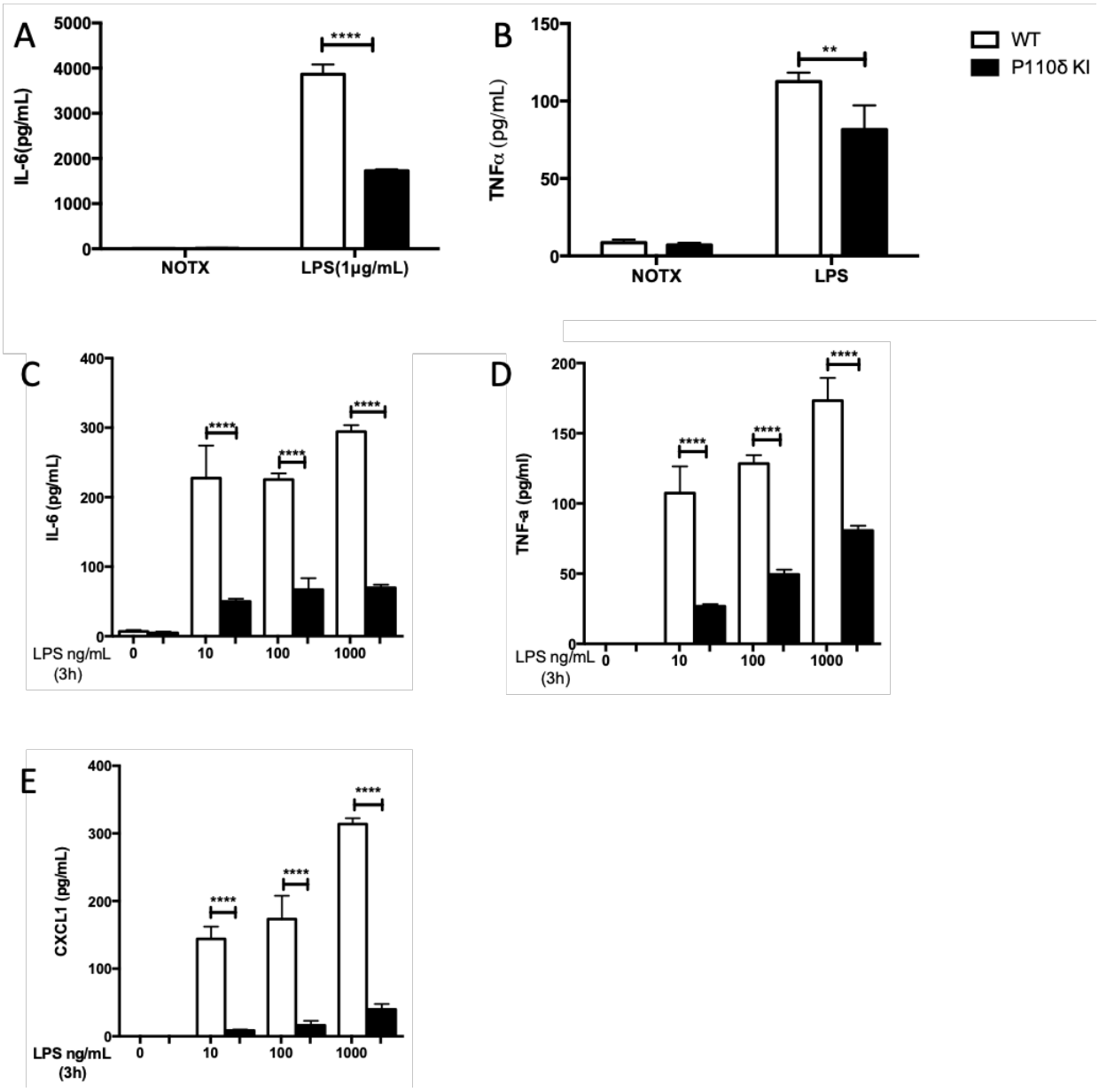


Figure 7: P110δ^{D910A} myeloid cells have a defect in cytokine and chemokine production following LPS stimulation.

Bone marrow derived macrophages (BMDM; A, B) or neutrophils (BMDN; C, D, E) from wild type (WT) and P110δ^{D910A} mice were stimulated with LPS. The levels of IL-6 (A, C), TNF-α (B, D) and CXCL1 (E) in the culture supernatant fluids were determined by ELISA. **, $p<0.01$; ****, $p<0.0001$.

3.5 CAL101 treated macrophages and neutrophils are impaired in their ability to produce proinflammatory cytokines like IL6 and TNFα

Next, I wanted to investigate the effect of CAL101 treatment on proinflammatory cytokine production in macrophages and neutrophils given the results seen in section 3.4. I isolated and grew BMDM and BMDN, treated them with CAL101 at different concentrations (1,10) μM for 4 hours before stimulating them with LPS from *E. coli* or *P. aeruginosa* and nigericin. Supernatant was collected; IL-6 and TNFα cytokine levels were measured by ELISA. BMDM (Fig. 8A, B) and BMDN (Fig. 8C, D) that were treated with CAL101 had a defect in TNFα and IL-6 production when compared to their WT counterparts irrespective of the LPS and the concentration of CAL101 used. This is consistent with the result gotten from the $p110\delta^{D910A}$ macrophages and neutrophils when compared to WT in Figure 7.

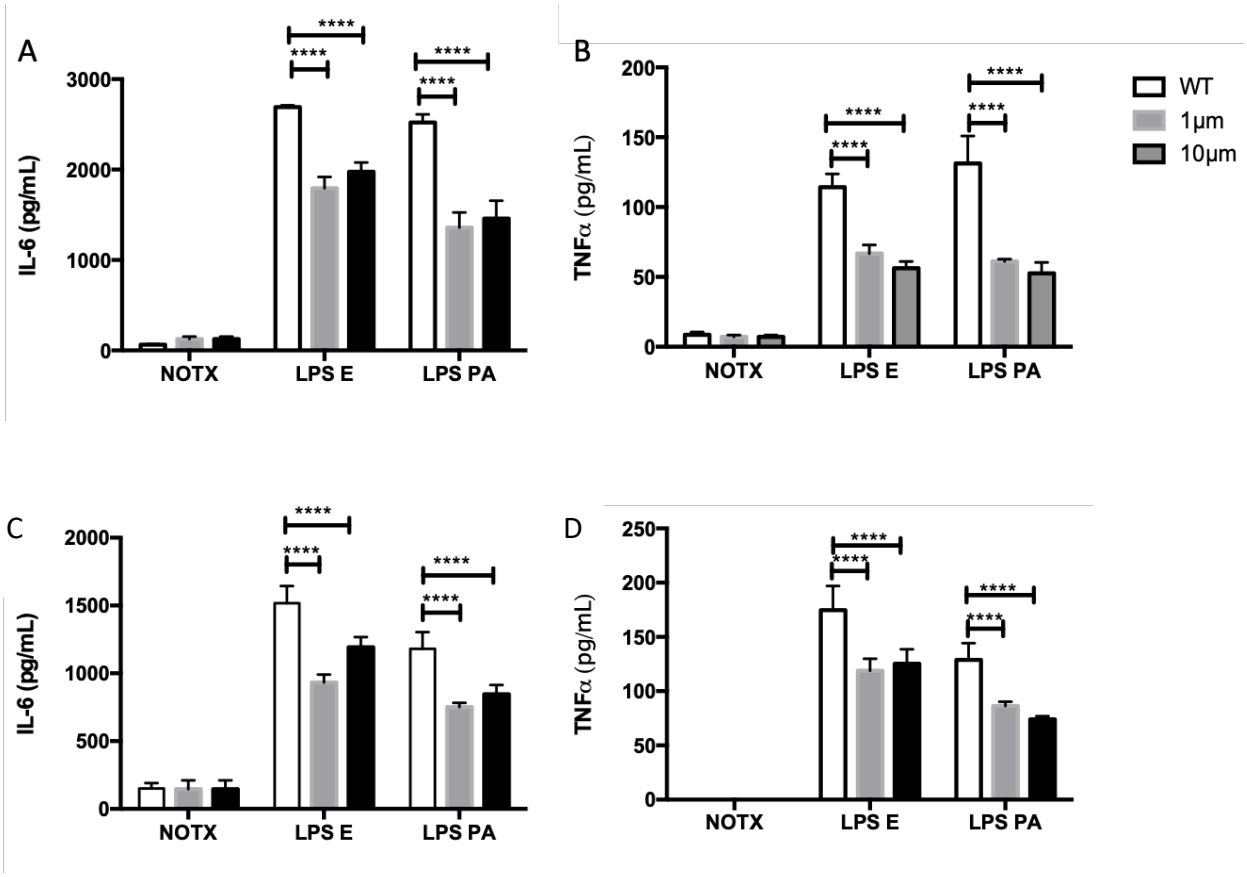


Figure 8. CAL101 treated macrophages and neutrophils are impaired in their ability to produce proinflammatory cytokines like IL6 and TNFα

BMDM and BMDN from wild type (WT) mice were treated with varying concentrations of CAL101(1,10) μM for 4 hours before stimulation with LPS from *E. coli* and *P. aeruginosa* (A, B). The levels of IL-6 (A, C) and TNF-α (B, D) in the culture supernatant fluids were determined by ELISA. ****, $p<0.0001$.

3.6 P110δ isoform of PI3K is dispensable for NLRP3 inflammasome response in neutrophils but not macrophages.

The inflammasome facilitate the maturation of pro-inflammatory cytokines such as IL-1β. Figures 4, 5 shows that P110δ is important for pro-inflammatory response not requiring the inflammasome in both macrophages and neutrophils. Next, I wanted to investigate if P110δ is critical for inflammasome response in macrophages and neutrophils. BMDMs and BMDNs were isolated from both WT and P110δ^{D910A} mice, treated with LPS and nigericin as described in the materials and methods section and the supernatant was collected and used for ELISA. I observed that p110δ deficient macrophages were defective in their ability to produce IL-1β (Fig. 9A) as observed for IL-6 and TNF-α(Fig. 7, 8 (A, B)) when compared to their WT counterparts. Surprisingly, P110δ deficient neutrophils produce similar amount of IL-1β as WT neutrophils and this was very different from what was observed for other proinflammatory cytokines including IL-6 and TNF-α(Fig. 7,8 (C, D)). To validate this, I used CAL101 to selectively inhibit P110δ in WT BMDM (Fig. 9C) and BMDN (Fig. 9C, D). This was then followed by LPS and Nigericin or ATP treatment to activate the inflammasome. This led me to conclude that inflammasome signalling in macrophages requires p110δ signaling but this pathway is dispensable in neutrophils.

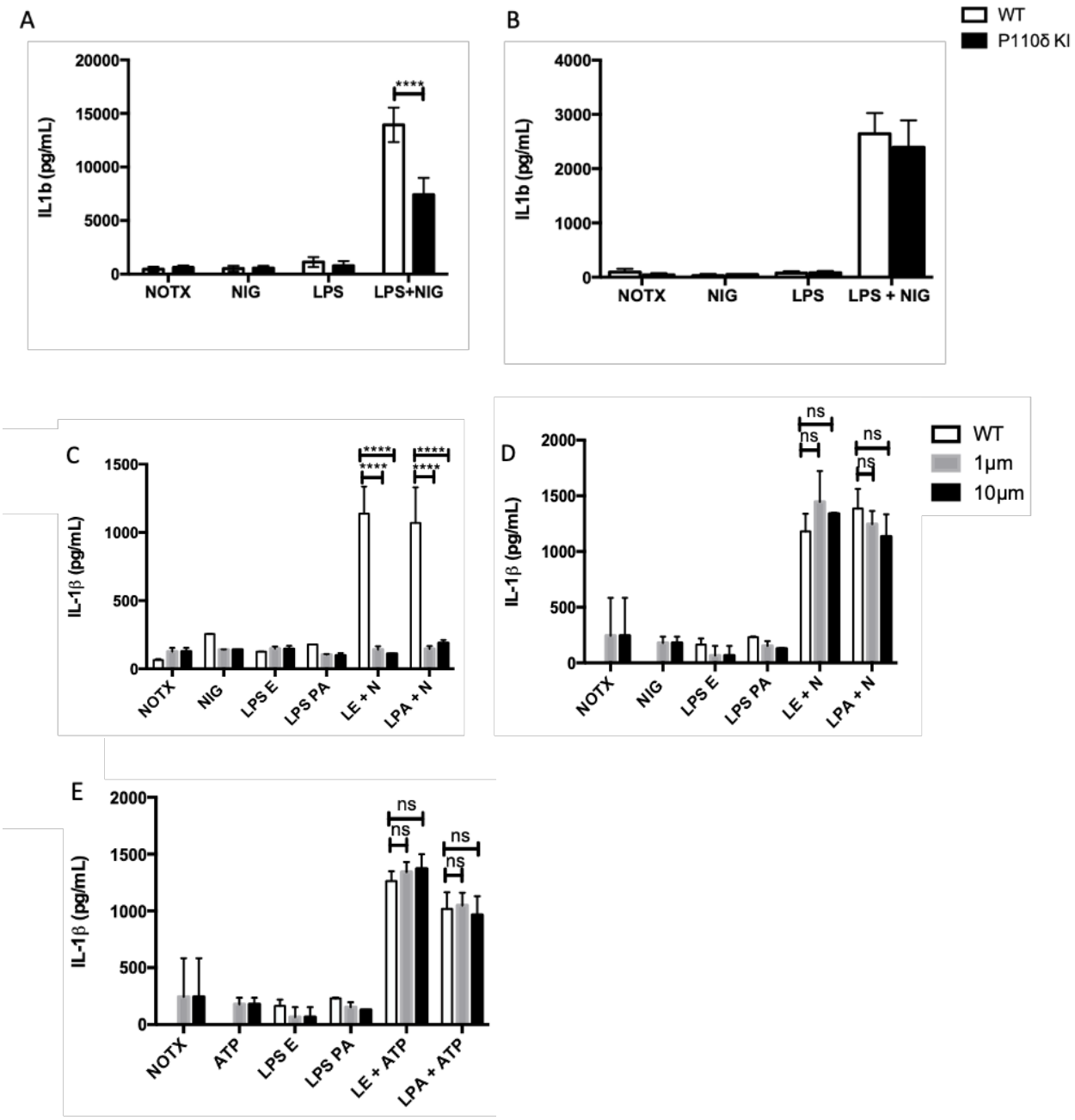


Figure 9. Inflammasome activation in macrophages require P110δ signaling but this pathway is dispensable in neutrophils.

Bone marrow-derived macrophages (BMDM; A) or neutrophils (BMDN; B) from wild type (WT) and P110δ^{D910A} mice were stimulated with four different treatments – Nigericin only, LPS (*E. coli*) only, LPS + Nigericin and no treatment (which acted as a control). CAL101 treated BMDM (C) and BMDN (D, E) were stimulated with LPS (*E. coli* and *P. aeruginosa*) and Nigericin or ATP. The level of mature IL-1β in the culture supernatant fluid was determined by ELISA. Results are a representative of four different experiments. ns = not significant; ****, $p<0.0001$.

3.7 P110δ^{D910A} the expression of inflammasome genes in macrophages and neutrophils is similar in WT macrophages and neutrophils from WT and P110δ^{D910A} mice.

I observed a significant difference in IL-1β production by macrophages and not neutrophils from WT and P110δ^{D910A} mice (Fig 9), so I sought to investigate if P110δ deficient macrophages and neutrophils were expressing the inflammasome complex genes such as pro- IL-1β, pro-caspase1, NLRP3, ASC differently. BMDMs and BMDNs were stimulated with LPS and nigericin as described in the material and methods section and mRNA was extracted, converted into cDNA and used for real-time PCR analysis. Consistent with the IL-1b ELISA result, there was no significant difference between the expression of pro- IL-1β, pro-caspase1, NLRP3, ASC mRNA in P110δ^{D910A} and WT macrophages (Fig. 10A). In contrast, P110δ^{D910A} neutrophils seem to express slightly more procaspase1 and NLRP3 mRNA levels than WT neutrophils (Fig. 10B). However, there was no significant difference in the expression of ASC and pro- IL-1β mRNA by WT and P110δ^{D910A} neutrophils Fig. 10B).

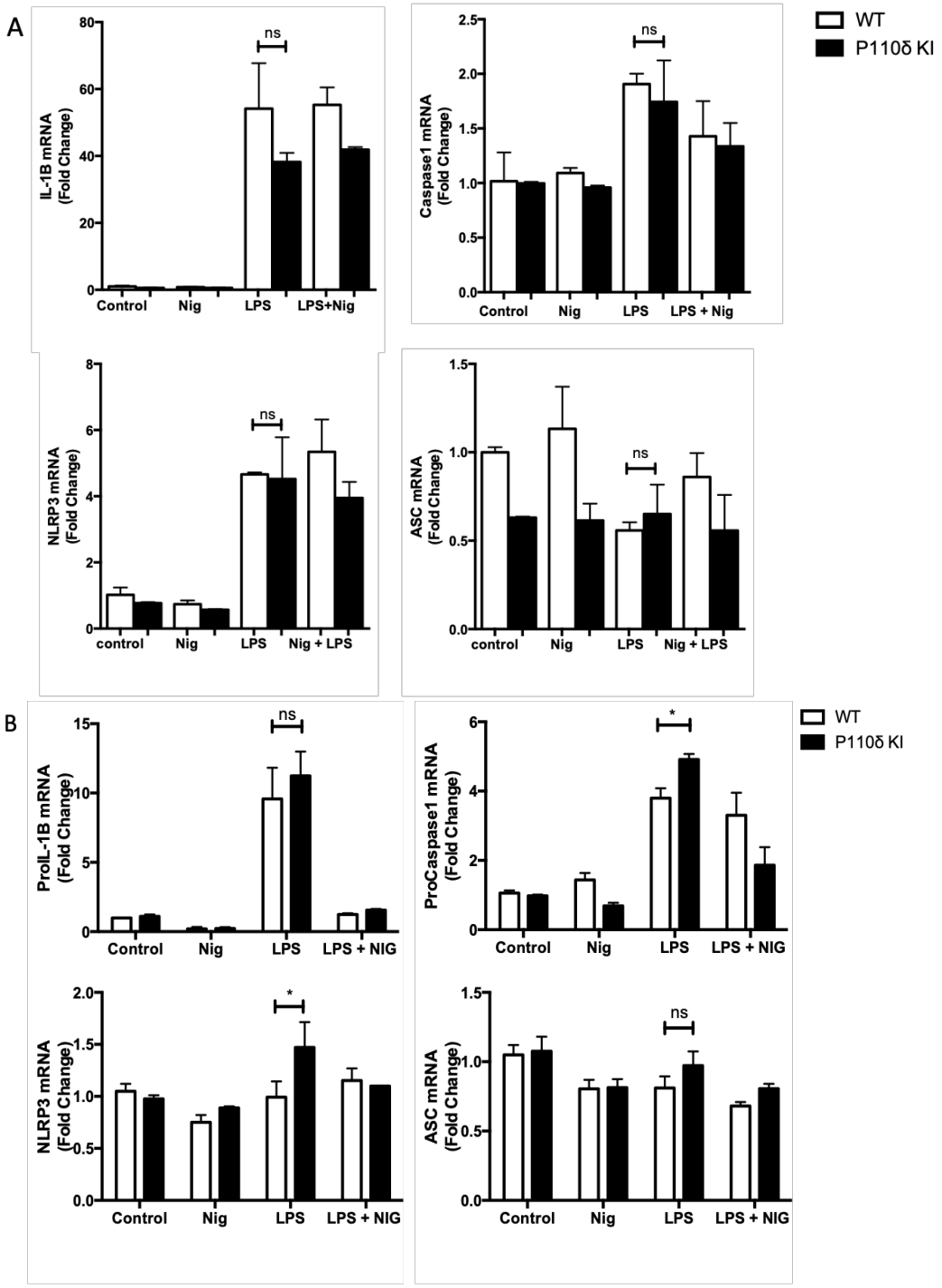
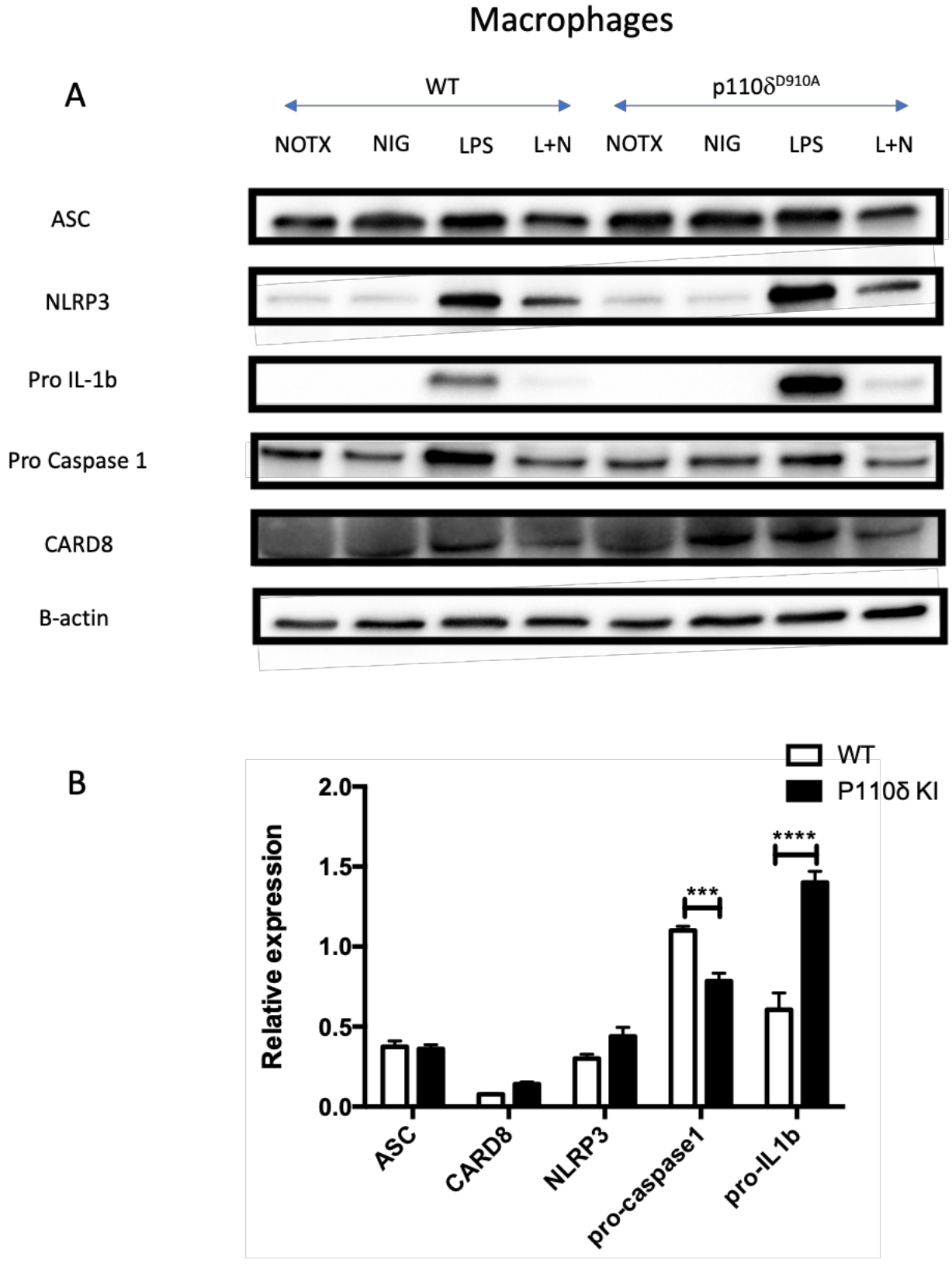


Figure 10. P110δ^{D910A} macrophages express inflammasome genes similarly to WT macrophages, which is not necessarily the case with neutrophils.
Bone marrow derived macrophages (BMDM; A) or neutrophils (BMDN; B) from wild type (WT) and P110δ^{D910A} mice were stimulated with four different treatments – Nigericin only, LPS only, LPS + Nigericin and no treatment which acted as a control. This was followed by RNA isolation and cDNA preparation which was later used for quantifying the level of pro-IL1β, pro-caspase1, NLRP3, ASC gene expression by qPCR and was expressed as Fold change. ns = not significant; *, $p<0.05$.

3.8 P110δ isoform of PI3K regulates procaspase 1 production in macrophages but not in neutrophils.

Although there appear to be little or no differences in the expression of inflammasome mRNA in macrophages and neutrophils from WT and P110δ deficient mice, it is conceivable that differences may exist in the protein levels because of differences in translational regulation. Therefore, I sought to investigate whether P110δ deficient macrophages and neutrophils were expressing different levels of the inflammasome complex proteins (such as ASC, NLRP3, pro-IL-1β, pro-caspase1) and its negative regulators (such as CARD8). BMDM and BMDN were stimulated with LPS and nigericin and cell lysates were prepared and used for western blot. I observed that P110δ deficient macrophages showed a significant decrease in the expression of pro-caspase1 (the enzyme that cleaves pro-IL-1β into mature IL-1β) and increased expression of pro-IL-1β when compared to WT macrophages (Fig. 11A, B). However, there was no significant change in ASC, NLRP3 and CARD8 expression (Fig. 11A, B). In contrast to macrophages, P110δ deficient neutrophils maintained similar expression of inflammasome related proteins similar to their WT counterparts (Fig. 11C, D). The decrease observed in the expression of pro-caspase1 in P110δ deficient macrophages would most likely translate to less caspase1 enzyme available. This would be the rate limiting step as less caspase 1 expression would lead to less mature IL-1β cleaved, which would explain the observed effect of P110δ signaling in macrophages. In contrast, since the expression of pro-caspase1 in P110δ deficient neutrophils doesn't differ from that observed in WT neutrophils, IL-1β expression would remain the same. In conclusion, P110δ isoform of PI3K regulates procaspase 1 production in macrophages but not in neutrophils.


Macrophages
A
WT
p110δD910A
NOTX
NIG
LPS
L+N
NOTX
NIG
LPS
L+N
ASC
NLRP3
Pro IL-1b
Pro Caspase 1
CARD8
B-actin
B
WT
P110δ KI
Relative expression
2.0
1.5
1.0
0.5
0.0
***
****
ASC
CARD8
NLRP3
pro-caspase1
pro-IL1b

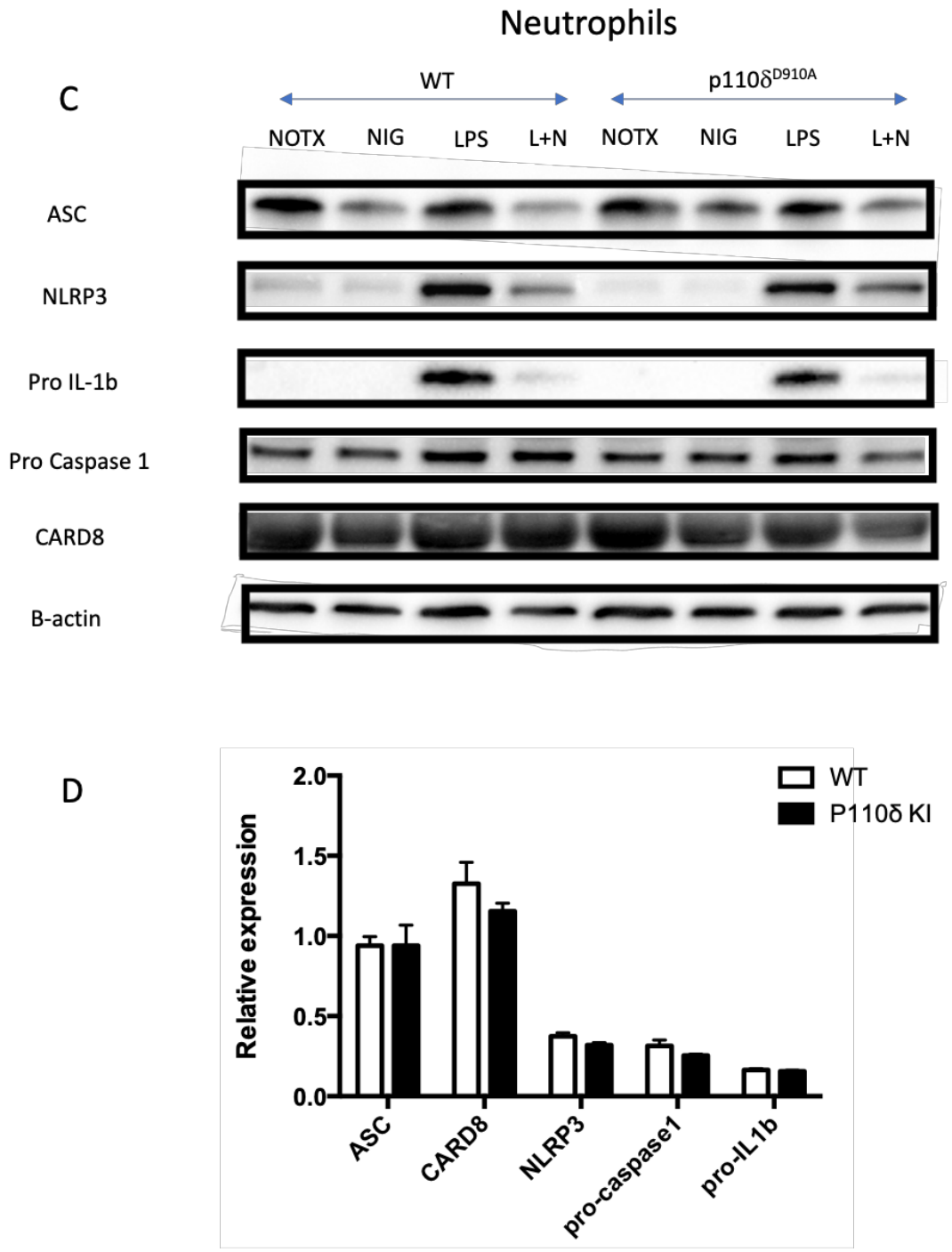


Figure 11. P110δ isoform of PI3K regulates procaspase 1 production in macrophages but not in neutrophils.

Bone marrow derived macrophages (BMDM; A and B) or neutrophils (BMDN; C and D) from wild type (WT) and P110δ^{D910A} mice were stimulated with with or without LPS in the presence or absence of Nigericin. The cell lysate was prepared and used for western blot to probe for the expression of ASC, NLRP3, pro- IL-1β, pro-caspase1, CARD8 proteins. ***, $p<0.001$, ****, $p<0.0001$.

3.9 Berenil downregulates LPS induced proinflammatory cytokine production and inflammasome response while upregulating anti-inflammatory cytokine production in bone marrow derived macrophages.

Berenil can function as an immunoregulatory agent. According to Kuriakose 2014, Berenil can downregulate proinflammatory cytokine production in macrophages by downregulating the phosphorylation of MAPKs, STATS, NFKβ and upregulating SOCS phosphorylation(39). However, it's role in inflammasome is yet to be determined. To investigate this, I isolated BMDMs and treated them with Berenil prior to stimulation with LPS and nigericin. I observed that Berenil treatment downregulated IL-1β production, which is regulated by the inflammasome response (Fig. 12A). In addition, and consistent with previous reports (39), Berenil pre-treatment also downregulated the production of IL-6, TNF-α, and IL-12p40 (Fig. 12B, C, E), cytokines that do not require the inflammasome for maturation. In contrast, IL-10, which is an anti-inflammatory cytokine, was upregulated by Berenil treatment (Fig. 12D). Collectively, these findings indicate that Berenil may be targeting the inflammasome response in a specific manner that is distinct from its effect on production of proinflammatory cytokines.

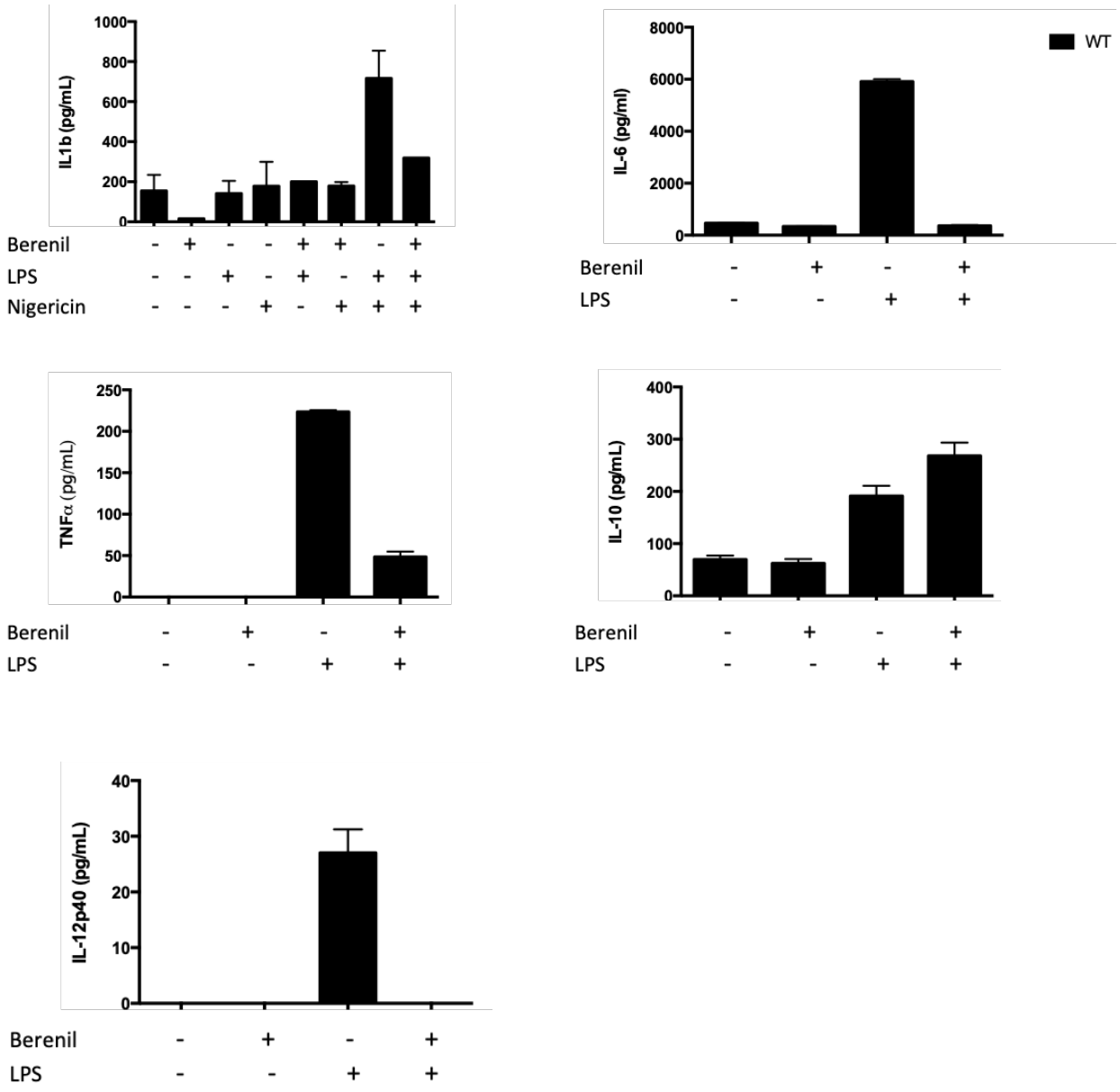


Figure 12. Berenil downregulates inflammasome activity and other proinflammatory cytokines but upregulates anti-inflammatory cytokine in macrophages.
WT Bone marrow derived macrophages (BMDM) were treated with Berenil (10μg/mL) for four hours prior to LPS and nigericin stimulation, then collected their supernatant for IL-1β, IL-6, TNF-α, IL-12p40 and IL-10 ELISA.

3.10 Berenil downregulates LPS-induced proinflammatory cytokine production and inflammasome response while upregulating anti-inflammatory cytokine production in bone marrow-derived neutrophils.

The preceding result showed that Berenil downregulates LPS-induced cytokine production and inflammasome response in macrophages while upregulating anti-inflammatory response. Next, I wanted to investigate if Berenil has a similar effect on neutrophils. I isolated BMDN and treated them with Berenil prior to LPS and nigericin stimulation, then collected their supernatant for ELISA. As with macrophages, I observed that Berenil treatment downregulated IL-1β production (which is regulated by the inflammasome) (Fig. 13A). Berenil treatment also downregulated IL-6 and TNF-α production (Fig. 13B, C) which do not require the inflammasome for production. As with macrophages, IL-10 which is an anti-inflammatory cytokine, was upregulated by Berenil treatment (Fig. 13D). IL-12p40 was undetectable in bone marrow-derived neutrophils. This observation indicates that Berenil affects the inflammasome and proinflammatory cytokine responses in neutrophils and macrophages similarly.

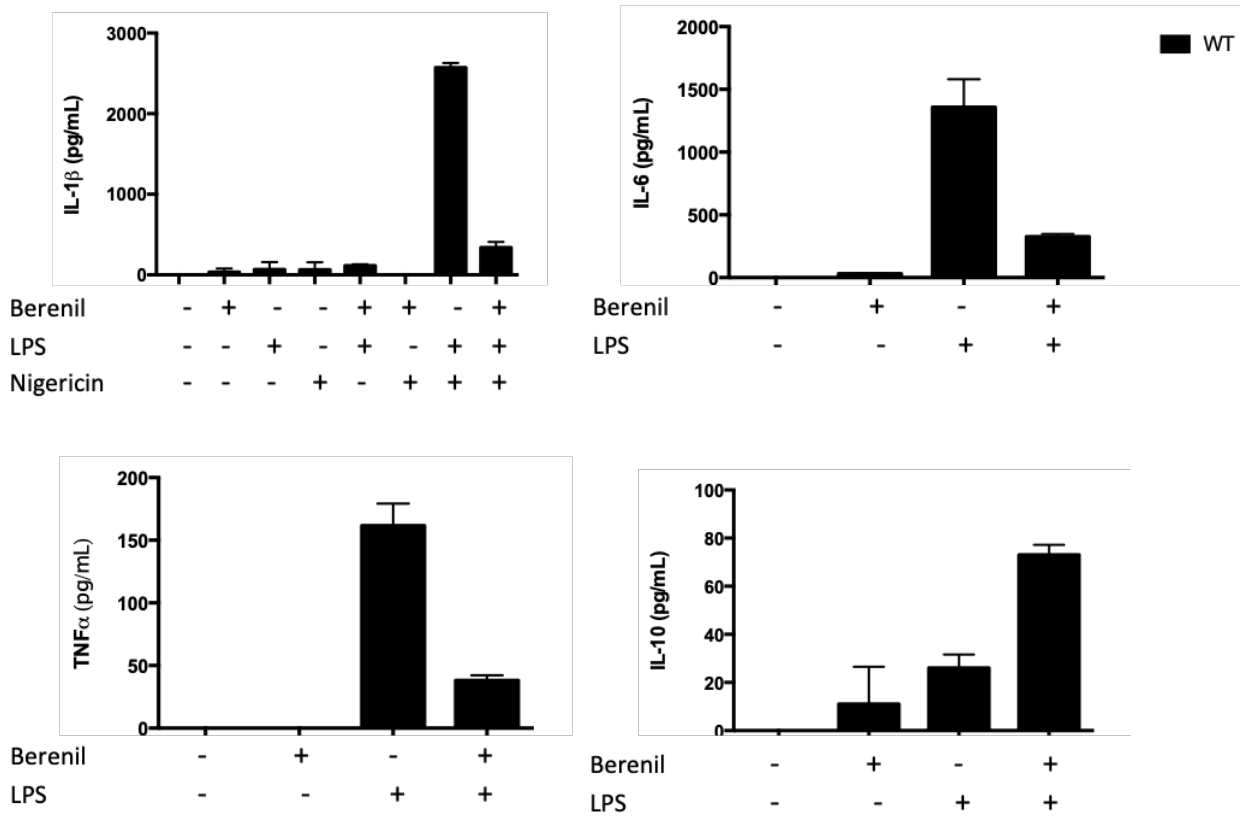


Figure 13. Berenil downregulates inflammasome activity and other proinflammatory cytokines but upregulates anti-inflammatory cytokine in Neutrophils.
WT Bone marrow derived neutrophils (BMDN) were treated with Berenil (10 μg/mL) for four hours prior to LPS and nigericin stimulation. The culture supernatant fluids were collected and assayed for IL-1β, IL-6, TNF-α, IL-12p40 and IL-10 ELISA.

3.11 Qualitative evidence suggests that P110δ is critical for NET formation.

P110γ signaling has been shown to be critical for NET formation following Leishmania infection(249), but little is known about the role of P110δ in NET formation. Therefore, to investigate the role of P110δ in NET formation, BMDN were isolated from both WT and $P110\delta^{D910A}$ mice. In addition, BMDN were isolated from WT and treated with the P110δ selective inhibitor, CAL101. NET formation was induced with PMA and/or LPS (from *E. coli* or *P. aeruginosa*). Immunofluorescence staining was performed for elastase (orange) and histone H3 (green). DAPI dye (blue) was used to stain the nuclei. Result was visualized by confocal microscopy. I observed reduced NET formation in $P110\delta^{D910A}$ neutrophils compared to WT neutrophils. I also observed reduced NET formation in CAL101 treated neutrophils compared to WT neutrophils. This indicates that P110δ is critical for NET formation following PMA and/or LPS stimulation. In addition, LPS from *P. aeruginosa* produced more NETs than LPS from *E. coli*. This implied that the virulence of the bacteria LPS can be influence NET formation in the neutrophils.

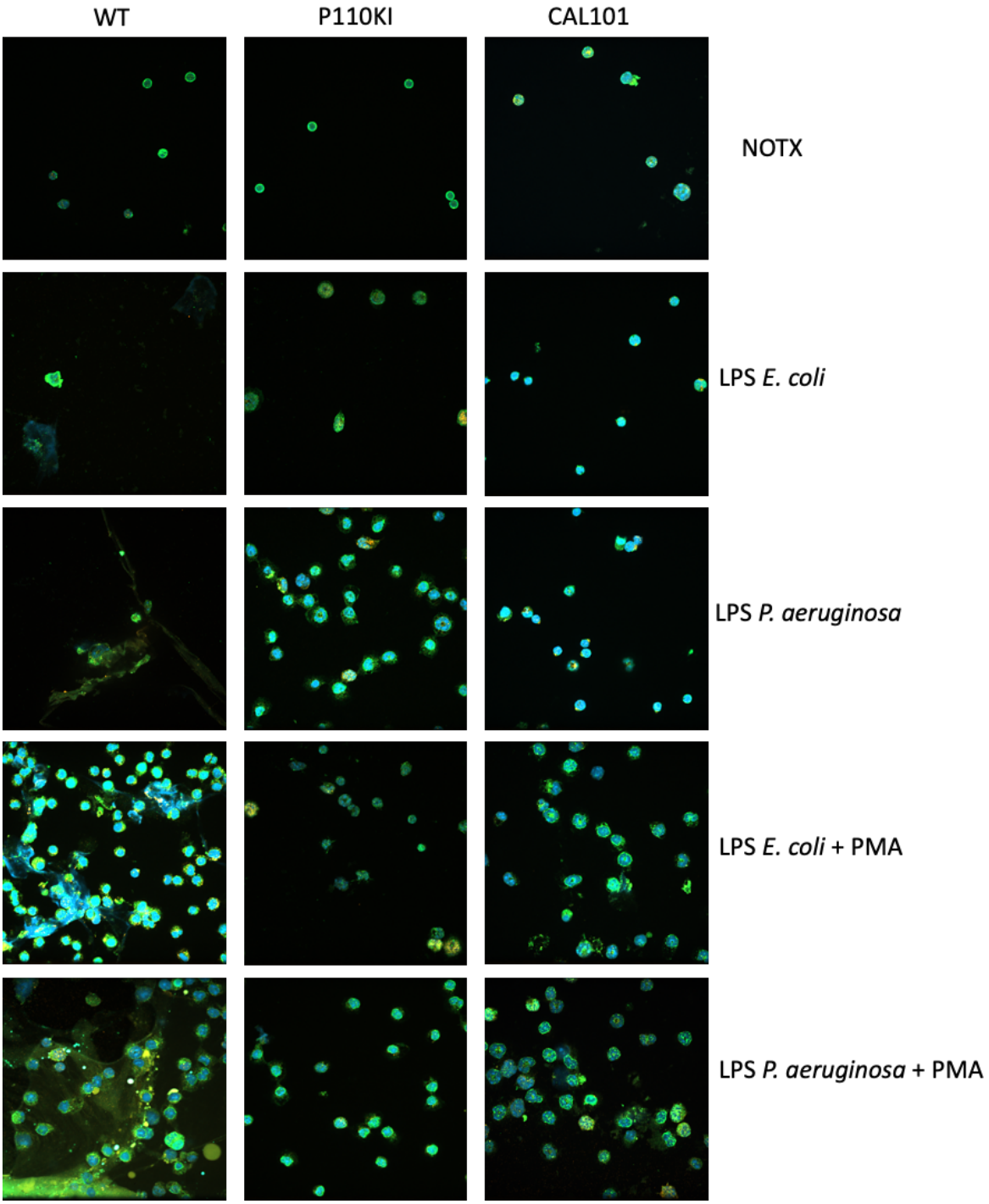


Figure 14. NET formation is reduced in P110δ^{D910A} neutrophils, CAL101 treatment reduced NET formation, and LPS from *P. aeruginosa* is capable of inducing more NET than LPS from *E. coli.*

Bone marrow derived Neutrophils were incubated on Poly-L-Lysine coated coverslips for 5 hours with or without CAL101(1μM) in the presence of PMA (100 nM) and LPS (1mg/mL) from *E. coli* or *P. aeruginosa*. NET formation was assessed by staining with anti-Histone H3 (green) and anti-Elastase (orange) antibodies and the images were assessed by confocal microscopy. DAPI (blue) staining was included to visualize cell nuclei.

3.12 Quantitative evidence suggests that P110δ is critical for NET formation.

Following my observation that P110δ is critical for NET formation where NET formation was assessed via immunofluorescent microscopy (Fig 14), I went ahead to verify this using a quantitative measure of dsDNA known as picogreen assay. To achieve this, BMDN were isolated from both WT and P110δ^{D910A} mice. In addition, BMDN were isolated from WT and treated with the P110δ selective inhibitor, CAL101. NET formation was induced with PMA and/or LPS (from *E. coli* or *P. aeruginosa*). The cell culture was used to perform the picogreen assay according to section 2.15 and a fluorescent microplate reader was used to determine the amount of dsDNA fluorescence present. As it was with the confocal microscopy result in section 3.11, I observed reduced NET formation in P110δ^{D910A} neutrophils compared to WT neutrophils. I also observed reduced NET formation in CAL101 treated neutrophils compared to WT neutrophils. This indicates that P110δ is critical for NET formation following PMA and/or LPS stimulation.

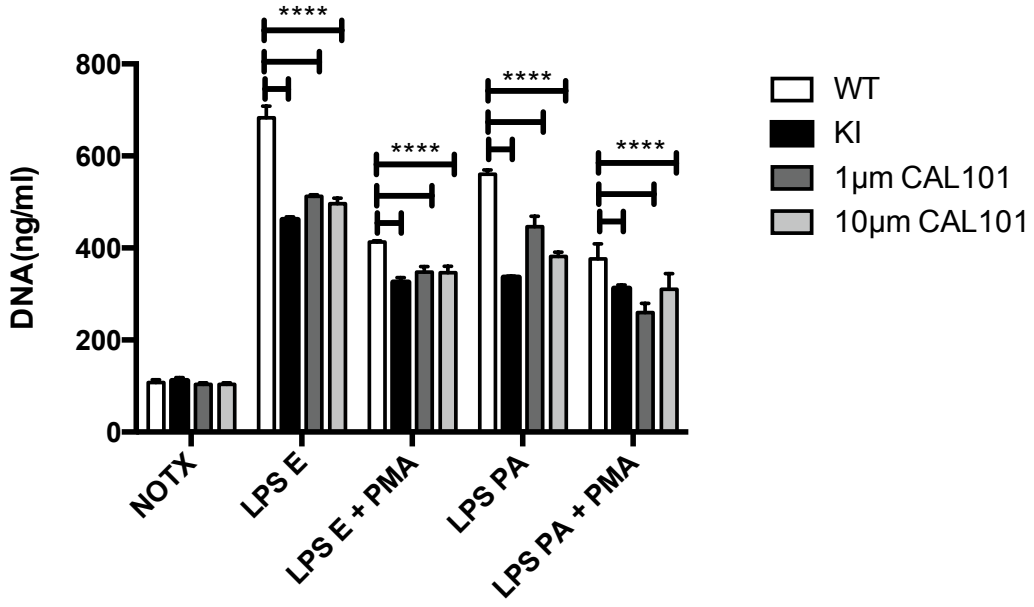


Figure 15. Quantitative evidence suggests that P110δ is critical for NET formation.
Bone marrow derived Neutrophils were treated with or without CAL101(1μM) in the presence of PMA (100 nM) and LPS (1mg/mL) from *E. coli* or *P. aeruginosa*. Net formation was assessed by picogreen assay and the resulting amount of dsDNA fluorescence present was determined with a fluorescent microplate reader. ****, $p<0.0001$.

3.13 P110δ signaling regulate NET formation in an animal model of sepsis.

Finally, I wanted to know if P110δ specifically regulates NET formation in an animal model of sepsis, which is a form of dysregulated inflammation. To induce Sepsis, WT and P110δ^{D910A} mice were challenged with 2.5mg/kg LPS from *E. coli* for 6 hours. This was immediately followed by peritoneal wash (PW) and blood collection. Immunofluorescence staining was performed for elastase (orange) and histone H3 (green), while DAPI dye (blue) was used to stain the nuclei. Result was visualized by confocal microscopy. Concurrently, picogreen assay was performed according to section 2.15 and a fluorescent microplate reader was used to determine the amount of dsDNA fluorescence present. I observed that P110δ^{D910A} mice is able to decrease the production of NET in both PW and blood sites following LPS challenge. This was observed both qualitatively with confocal microscopy (Fig. 16A) and quantitatively with picogreen assay

(Fig. 16B, C). These results confirm the *in vitro* data seen in Figure 14 and 15, suggesting that the absence of P110δ signaling leads to decreased Net formation *in vivo*.

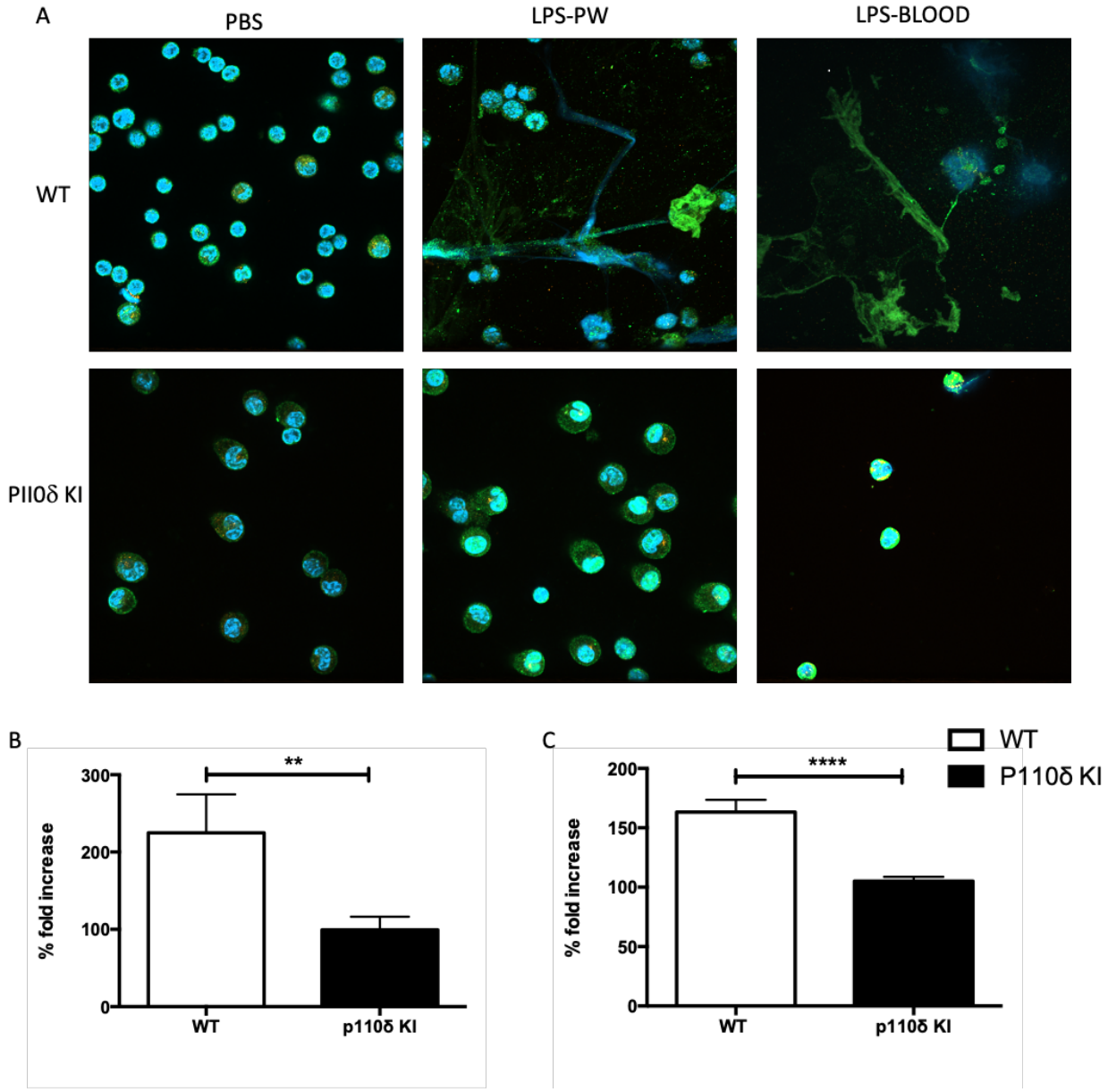


Figure 16. Absence of P110δ signalling leads to decreased NET formation *in vivo*. WT and p110δ^{D910A} mice were challenged with 2.5mg/kg LPS from *E. coli* for 6 hours. This was immediately followed by peritoneal wash (PW) and blood collection. NET formation was assessed by staining with anti-Histone H3 (green) and anti-Elastase (orange) antibodies and the images were assessed by confocal microscopy. DAPI (blue) staining was included to visualize cell nuclei (A). Alternatively, picogreen assay was used to quantify NET and the resulting amount of dsDNA fluorescence present was determined with a fluorescent microplate reader (B, C). **, $p<0.01$; ****, $p<0.0001$.

CHAPTER 4: DISCUSSION

4.1: GENERAL DISCUSSION

In recent years, Phosphatidylinositol 3-kinases has been recognized as an important protein responsible for influencing various activities of immune and non-immune cells, including proliferation, differentiation, and effector activities. Different studies are beginning to decipher the distinct cell specific roles for different Phosphatidylinositol 3-kinase subunits in immunity and inflammation (252).

The findings from this study clearly highlights the role played by the P110δ subunit of PI3K play in neutrophil migration, inflammasome activation and NET formation. To overcome limitations that arise when only one study approach is used and hence validate the observations reported here, I used both genetic and pharmacological inhibition of P110δ subunit of PI3K to investigate its role in neutrophil functions. My findings suggest that signaling via the P110δ isoform of PI3K plays an important role in regulating migration and NET formation but is dispensable for inflammasome response in neutrophils.

According to Lieschke, Keightley and Manley (2018), the physiological state of a neutrophil cell can affect its plasticity, migratory properties and maturation(253). Accelerated apoptosis can decrease neutrophil's lifespan and downregulate its function while delayed apoptosis can upregulate neutrophil's function and increase its lifespan(254). Neutrophils possess a variety of cell surface molecules such as CD11b and Ly6G for pathogen recognition, migration and differentiation(255,256). Therefore, my discovery that P110δ subunit of PI3K does not affect neutrophil's morphology, survival rate or surface molecule expression was critically important because any other differences observed in this study could not be attributed to structural differences, apoptosis or surface marker expression on neutrophils.

The P110δ isoform of PI3K have been shown to regulate the migration of NK, B and T cells(241,243,244). Likewise, studies have shown that other class 1A PI3Ks such as P110γ are involved in neutrophil migration(241,249). Although P110δ have been shown to play an important role in neutrophil functions, their direct involvement in neutrophil migration has not been established. Following exposure of bone marrow-derived neutrophils (BMDNs) to chemokines such as MIP1a and CXCL1, I observed that neutrophils from P110δ deficient mice exhibited impaired ability to migrate towards these chemokines. This was later found to be due to the low expression of chemokine receptors such as CXCR2 and CCR1, suggesting that p110d controls the expression of these receptors on neutrophils. This result correlated with the literature and is in line with my hypothesis as there are two main reasons cells would not migrate: either the cell's morphology has been compromised or the cell has lost its ability to respond to these chemokines(257,258). Collectively, these observations show that the P110δ isoform of PI3K is necessary for neutrophils to respond to chemokines and migrate towards them accordingly.

The production of proinflammatory cytokines such as IL-6, IL-1β, TNFα, etc. is a great indication that the immune system is able to boost the acute phase response and activate the endothelium(1,7). However, an excessive and unregulated production of these proinflammatory cytokines result in many inflammatory diseases (18). I observed that P110δ deficient macrophages as well as CAL101 treated WT macrophages were defective in their ability to produce IL-1β, a key proinflammatory cytokine who production is influenced by inflammasome activation. This was also the case with IL-6 and TNF-α, other proinflammatory cytokines not influenced by inflammasome activity. Surprisingly, P110δ deficient neutrophils produce similar amount of IL-1β as WT neutrophils and this was very different from what was observed with IL-6 and TNF-α both in the P110δ KI neutrophils and those treated with CAL101. This confirms

that indeed, the regulation of IL-1β by P110δ signaling is different from other pro-inflammatory cytokines in neutrophils. These findings suggest that signalling pathways can function differently in different immune cells, probably based on the cell's function, and time of stimulation. I noticed that neutrophils do not release IL-1β until after 2 - 4 hours of nigericin stimulation whereas macrophages can release IL-1β after 30 – 45 mins of nigericin stimulation. This might mean that IL-1β produced by macrophages and neutrophils might be needed at different times to exert different functions in the immune system.

According to Mulero et al. (2021), neutrophils prefer the use of serine proteases such as proteinase 3 and cathepsin to cleave matured IL-1β while macrophages prefer the use of inflammasome dependent pathways (259). My result show that P110δ signaling affects procaspase-1 which is the effector enzyme in macrophage inflammasome but does not affect procaspase-1 in neutrophils. These findings together suggest that P110δ signaling may not affect procaspase-1 expression in neutrophils because of their preferential use of serine proteases (and not the regular inflammasome pathway) to cleave IL-1β. Although not tested in this study, it is conceivable that P110δ signaling might not be affecting proteinase 3 and cathepsin expression. Notwithstanding, these speculations need to be further investigated.

I focused on NLRP3 inflammasome to study the impact of P110δ signaling on inflammasome activation inflammasome because NLRP3 is the best characterized inflammasome so far because it has a wide range of activators (260). I found that blocking P110δ signaling in macrophages decreased NLRP3 inflamamsome activation. Other studies have shown that P110δ signaling play a similar role in NLRP3 inflammasome in other cell types. Blocking P110δ signaling in epithelial cells improved fungal induced allergic inflammation by modulating NLRP3 inflammasome-associated IL-1β (261). However, this was not reflected in neutrophils.

Furthermore, my findings suggest that P110δ signalling uses a feedback loop to control inflammasome activation. In the P110δ deficient macrophages, I observed more production of proIL-1β, less production of caspase-1 and less production of matured IL-1β than in WT macrophages. These observations suggest that the low production of mature IL-1β feeds back to the macrophage to produce more proIL-1β. However due to reduced caspase-1 production, the produced proIL-1b cannot be cleaved leading to its accumulation in the cell. Therefore, caspase-1 is the rate limiting step. In contrast, WT macrophages can cleave its proIL-1β to matured IL-1β due to abundance of caspase-1, and this feedbacks to the cells to decrease the production of proIL-1β. I did not see these changes in neutrophils, confirming that P110δ signalling does not affect inflammasome activation in neutrophils.

Next, I investigated at what stage of inflammasome activation that P110δ signalling was acting. Procaspase-1 and proIL-1β protein expressions are affected after priming BMDM with LPS but were not affected in BMDN after LPS priming. Collectively, these findings suggest that P110δ signalling is acting at the priming stage of inflammasome activation.

Although there appear to be little or no differences in the expression of inflammasome mRNA in macrophages and neutrophils from WT and p110δ deficient mice, it is conceivable that differences may exist in the protein levels because of differences in translational regulation. Inflammasome protein expression is more impactful than its mRNA expression because many post-translational regulations such as NLRP3 ubiquitynation deubiquitylation, NLRP3 phosphorylation and dephosphorylation, Nitrosylation and sumoylation can occur before the protein is expressed(262).

Recently, Arowolo et al. demonstrate that Berenil has the ability to block responses induced by histamine and exerts other anti-inflammatory effects apart from its trypanocidal activity (38).

My findings suggest that Berenil treatment downregulated IL-1β production and other proinflammatory cytokines such as IL-6, TNF-α, IL-12p40 which do not require the inflammasome activation for their production. In contrast, IL-10 which is an anti-inflammatory cytokine, was upregulated by Berenil treatment in both neutrophils and macrophages. This finding confirms that Berenil is targeting the inflammasome response in a specific manner. Therefore, its impact on decreasing proinflammatory cytokines production was not due to nonspecific toxicity of the drug on the cells. My findings suggest that Berenil is acting at the inflammasome priming stage as well, since it is able to influence other cytokines that do not require the inflammasome for maturation. Thus, Berenil is a plausible therapy for inflammation and diseases associated with dysregulated inflammasome as it does not just decrease pro-inflammatory cytokines, but it enhances anti-inflammatory cytokines such as IL-10, making it an immunoregulatory agent. Previous reports by Kuriakose et al. showed that Berenil suppresses IL-6, IL-12 and TNF production in BMDM by regulating NF-κB p65 activity, mitogen-activated protein kinases (p38 and JNK), signal transducer and activator of transcription (STAT) proteins (STAT1 and STAT3) activities(39). Therefore, Berenil might be suppressing the inflammasome activity and upregulate IL-10 production via similar mechanisms; however, that would have to be validated with proper experimental studies.

Earlier studies found that P110γ have been linked to the regulation of ROS dependent NETosis in response to *Leishmania* infection by activating ERK (249). Although the mechanism is yet to be delineated, my findings suggest that NET formation is regulated by P110δ signalling both *in vitro* and *in vivo* in LPS-induced animal model of sepsis. This finding supports existing literature on the role of PI3K in regulating NET formation and NETosis (249). NET formation in the context of sepsis has been shown to be both protective and detrimental to the host depending

on the stage of sepsis. Some studies show that when mice were treated with DNase (which breaks down NETs), the onset of sepsis was accelerated(207). This suggests that NET formation is protective during sepsis and could explain why P110δ deficient mice which have impaired NETs formation show increased mortality following LPS-induced sepsis compared to their WT counterparts(247). In contrast, NET can damage the lungs and liver as the disease progresses by causing epithelial and endothelial cell death(208). However, the administration of DNase improved survival and decreased organ failure in a well-established sepsis condition(209). The regulation of NET by P110δ signalling can be useful based on the stage of sepsis according to these studies. The use of CAL101 as a drug therapy to treat sepsis can be administered in increasing dosage as the disease progresses to control NET formation and subsequently sepsis condition. However, more research has to be done to confirm these speculations.

One potential assumption is that the reduction in proinflammatory cytokine production, inflammasome response and NET formation observed in P110δ deficient mice could be as a result of reduced ability of their neutrophils to migrate appropriately to the site of inflammation or tissue insult. However, my *in vitro* studies have provided some evidence that even when you have equal number of neutrophils in culture; reduced IL-6 and TNF-α cytokines and NET formation is observed in P110δ deficient neutrophils while IL-1β remains the same. Therefore, the reduction in proinflammatory cytokine production, inflammasome response and NET formation observed in P110δ deficient mice would occur even when cell migration is not a variable. However, while impaired migration is most likely a factor, I have no sufficient evidence to unequivocally prove that cell migration defects does not worsen these observations.

4.2 CONCLUSION

In conclusion, my findings suggest that signaling via the p110δ isoform of PI3K plays an important role in regulating neutrophil migration and NET formation but is dispensable for NLRP3 inflammasome response in neutrophils, though signaling via the p110δ isoform of PI3K regulate NLRP3 inflammasome response in other myeloid cells such as macrophages.

4.3 SIGNIFICANCE OF STUDY

This study unravels the role of the p110δ isoform of PI3K signaling in NET formation, the induction of proinflammatory cytokine production, and inflammasome response in neutrophils. This study also proposes novel strategies for controlling inflammatory diseases. P110δ isoform of PI3K signaling could be a potential drug target for controlling inflammatory diseases such as sepsis, since CAL101 has shown reduced inflammatory response *in vitro*.

4.4 LIMITATION

1. Due to species differences, the findings in mouse models are not always translatable in humans. Studies should be conducted using human neutrophils to determine whether this isoform of PI3K plays similar role in inflammasome and net formation in neutrophils.
2. Another limitation is the use of dsDNA assays to quantify NET formation. Apart from NET formation, the active metabolic secretion of DNA from cells and other forms of cell death such as necrosis can result in the release of circulation cell free DNA(263). To make sure NET is properly assessed *in vivo*, NETosis markers such as NE, MPO, and citrullinated histone H3 can be tagged with GFP, YFP or RFP and used to track how much NET is formed outside the cell.

4.5 FUTURE DIRECTIONS

Use live bacteria to study inflammatory response in sepsis model

I have previously used LPS found on gram negative bacteria's cell wall to simulate what would have naturally occurred with a bacterial infection in all my findings. The use of live bacteria to study the inflammatory response in a sepsis model in both WT and p110δ^{D910A} mice would further emphasize the role of p110δ in disease progression as it relates to NET formation, cytokine profile and the inflammatory phenotype in different tissues due to the fact that it would simulate exactly the infection process – as infectious agents such as bacteria is more likely to invade animals rather than LPS.

Check the impact of treating sepsis induced mice with CAL101

My finding shows that CAL101 – the specific inhibitor of P110δ subunit of PI3K signaling – was able to reduce inflammatory response *in vitro*. Administration of CAL101 drug in a dose dependent manner to a WT mouse that has been induced with sepsis would give us some insight into whether drug therapy targeting P110δ signalling can impact survival, NET formation and inflammasome activity *in vivo.*

Delineate the mechanism of P110δ regulated NET formation

Other studies have linked ERK, PKC, Ca^{2+}, ROS, NADPH oxidase, PAD4, autophagy and others to the mechanism of NET formation in different systems(198,202,203,249). To know definitively the mechanism of P110δ regulated NET formation in neutrophils, loss or gain of function studies can be done on ERK, PKC, Ca^{2+}, ROS, NADPH oxidase, PAD4, autophagy and

others to identify those that are responsible. Also, their individual inhibitors can be employed to validate this.

Discover other molecules associated with sepsis induced NET formation

I found that P110δ signalling regulates LPS-induced sepsis in mice. It would be interesting to study the cytokines, granular and nuclear molecules associated with sepsis-induced NET formation at different sites. More so, studying the impact of the absence of P110δ signaling on the production of these cytokines and molecules would give us more insight into the regulation of NET formation by P110δ signalling.

Understand the systemic effects of sepsis induced NET formation

When sepsis was induced intra-peritoneally by LPS, I found that there was a defect in NET formation in both peritoneal wash and peripheral blood of mice deficient in P110δ signalling. I am interested in investigating whether P110δ signalling can affect NET formation in a distal site of inflammation such as the lungs. This would provide more insight into the systemic influence of P110δ signalling on the inflammatory response.

Determine the impact of P110δ signaling on other inflammasomes

The impact of P110δ signaling on inflammasome activation was studied in the NLRP3 inflammasome because it is the best characterized inflammasome so far as a result of its range of activators (260). Further studies would focus on other inflammasome types to determine whether they are affected by P110δ signalling.

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