Identifying Novel Regulators of Neutrophil Survival and Function

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Abstract

Persistent and inappropriate neutrophilic inflammation underpins many inflammatory conditions, including chronic obstructive pulmonary disease (COPD). Prolonged neutrophil survival, along with defects in neutrophil function, are believed to be key contributors to pathophysiology in COPD and other inflammatory diseases. Uncovering the mechanisms underpinning the dysregulation in neutrophil apoptosis and function may allow development of novel therapeutic strategies for inflammatory diseases.

In this study, circulatory neutrophils from COPD patients were found to have a modest phagocytic defect. The phagocytic capacity in healthy neutrophils was not modulated by key pro-inflammatory factors (IFN-γ, mROS, and hypoxia).

Protein kinase cascades play central roles in numerous cellular functions including cell survival, and kinase inhibitors are increasingly being considered as therapeutics in multiple pathologies. To identify therapeutically targetable novel neutrophil apoptosis regulator(s), a protein kinase inhibitor library was screened in neutrophil apoptosis assays. Screening identified 11 compounds that robustly increased neutrophil apoptosis ≥ 2-fold over control and have greatest specificity for their kinase targets. The family of ErbB RTKs was identified as frequent targets of the identified compounds. Inhibition of ErbB1 and ErbB2 by selective small molecule inhibitors, Erbstatin analog and Tyrphostin AG825 respectively, increased apoptosis in neutrophils from healthy subjects and COPD patients, and also overrode the effects of LPS-, GMCSF-, and cAMP-mediated neutrophil survival. Tyrphostin AG825 reduces GMCSF induced AKT-phosphorylation, suggesting that ErbB2-driven neutrophil survival may be transduced via the PI3K/AKT pathway. Tyrphostin AG825 significantly reduces neutrophil burden at the site of inflammation in an *in vivo* zebrafish tail injury model and also significantly elevates neutrophil apoptosis and efferocytosis of apoptotic neutrophils in the lung in an acute lung injury murine model.

These findings may identify druggable protein kinase dependent pathways that will allow the development of novel therapeutics for inflammatory disease.

Publicaitons arising from this thesis

Expected publication

1. Defining the functional neutrophil kinome reveals ErbB kinases as potential therapeutic targets in inflammation. **Atiqur Rahman,** Katherine M Henry, Claudia Tulotta**,** David Sammut, A. A. Roger Thompson, Julien J. Y. Rougeot, Abigail Reese, Kathryn Higgins, Caroline Tabor, Ian Sabroe, William J. Zuercher, Caroline O. Savage, Annemarie H. Meijer, Moira K. B. Whyte, David H. Dockrell, Stephen A. Renshaw, Lynne R. Prince

Published abstracts

1. Protein kinase inhibitor compound screen reveals ErbB family kinases as regulators of neutrophil apoptosis in the context of inflammatory diseases. **Atiqur Rahman**, Katherine Henry, David Sammut, A. A. Roger Thompson, Bill Zuercher, Moira Whyte, David Dockrell, Stephen Renshaw, Lynne Prince. The Society of Leukocyte Biology's 49th Annual Meeting, Verona, Italy. 15-17th Sept 2016. (**Oral presentation)**
2. Using protein kinase inhibitor compounds to drive neutrophil apoptosis in the context of chronic inflammation. **Atiqur Rahman**, Kathrine Henry, David Sammut, Richard Budd, ,Moira Whyte, David Dockrell, Stephen Renshaw, Lynne Prince. Cold Spring Harbor Laboratory Cell Death Meeting, September 15 - 19, 2015.
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Table of Contents

List of Figures

List of Tables

Page

List of abbreviations

A	ABB	Annexin Binding Buffer
	AKT	Protein Kinase B (PKB)
	ALCAM	Activated Leukocyte Cell Adhesion Molecule
	ALI	Acute Lung Injury
	ANOVA	Analysis of Variance
	Apaf1	Apoptotic Protease Activating Factor 1
	APC	Allophycocyanin
	ARDS	Acute Respiratory Distress Syndrome
	ATP	Adenosine Triphosphate
B	BAL	Bronchoalveolar Lavage
	BAD	Bcl-2-associated death promoter
	BAK	Bcl-2 homologous antagonist/killer
	BAX	Bcl-2-associated X protein
	Bcl-2	B-cell Lymphoma 2
	BPI	Bactericidal Permeability Increasing Protein
	BTC	Betacellulin
C	cAMP	Cyclic Adenosine Mono Phosphate
	CDK	Cyclin-dependent Kinase
	CGD	Chronic Granulomatous Disease
	COPD	Chronic Obstructive Pulmonary Disease
	CR	Complement Receptor
	CREB	cAMP Responsive Element Binding protein
	CRP	C-reactive Proteins
	CXCL	Chemokine-CXC-Ligand
	CXCR	CXC-chemokine Receptor
D	DC	Dendritic Cell
	DHE	Di-hydroethidium
	DISC	Death Inducing Signalling Complex
	DMSO	Dimethyl Sulfoxide
	DYRK1B	Dual Specificity Tyrosine-Phosphorylation-Regulated kinase 1B

E	EDTA	Ethylenediaminetetraacetic acid
	EGF	Epidermal Growth Factor
	EGFR	Epidermal Growth Factor Receptor
	EPG	Epigen
	EPR	Epiregulin
	ErbB	Epidermal Growth Factor Receptor
	ERK	Extracellular Signal Regulated Kinase
	ESAM	Endothelial cell-Selective Adhesion Molecule
F	FACS	Fluorescence-Activated Cell Sorting
	FADD	Fas-Associated Death Domain
	FBS	Fetal Bovine Serum
	FDA	Food and Drug Administration
	FEV1	Forced Expiratory Volume in the first second
	FIH	Factor-Inhibiting HIF
	fMLP	N-Formyl Methionyl-Leucyl-Phenylalanine
	FPRs	Formyl Peptide Receptors
	FSC	Forward Scatter
G	G6PD	Glucose-6-phosphate Dehydrogenase
	G-CSF	Granulocyte colony stimulating factor
	GFP	Green Fluorescence Protein
	GMCSF	Granulocyte-Macrophage Colony Stimulating Factor
	GMI	Geometric Mean Intensity
	GPCRs	G-Protein Coupled Receptors
	GSK	GlaxoSmithKline
	GSR	Glutathione Reductase
H	H & E	Hematoxylin & Eosin
	HBSS	Hank’s Balanced Salt Solution
	HC	Healthy Control
	hCAP	human Cathelicidin Antimicrobial Protein
	HIF1α	Hypoxia-Inducible Factor-1 alpha
	HO-1	Heme Oxygenase-1
	hpi	Hours Post Injury
	HREs	Hypoxia Responsive Elements

	HTS	High-Throughput Screening
I	IBD	Inflammatory Bowel Disease
	IC_{50}	50% Maximum Inhibitory Concentration
	ICAM	Intercellular Adhesion Molecule
	IFN-γ	Interferon-gamma
	IgG	Immunoglobulin G
	IL	Interleukine
	iNOS	Inducible Nitric Oxide Synthase
	ISP	Individual Study Plan
J	JAK	Janus Kinase
	JAM	Junctional Adhesion Molecule
L	LFA-1	Lymphocyte function-associated antigen-1
	LTA4H	Leukotriene A4 Hydrolase
	LTB4	Leukotriene B4
	LPS	Lipopolysaccharide
	LXA4	Lipoxin A4
M	MAPK	Mitogen Activated Protein Kinase
	MEK	Mitogen Activated Kinase/ERK kinase
	MFI	Mean Fluorescence Intensity
	MIP	Macrophage Inflammatory Protein
	MOI	Multiplicity of Infection
	MPO	Myeloperoxidase
	mROS	Mitochondrial Reactive oxygen Species
N	NADPH	Nicotinamide Adenine Dinucleotide Phosphate
	NE	Neutrophil Elastase
	NET	Neutrophil Extracellular Trap
	NF-κB	Nuclear Factor-Kappa B
	NGAL	Neutrophil Gelatinase-Associated Lipocalin’
	NK	Natural-Killer
	NO	Nitric Oxide
	NQO1	NADPH: Quinone Oxidoreductase 1

	NRE	Nrf2 Responsive Elements
	NRES	National Research Ethics Service
	Nrf2	Nuclear factor erythroid-2–Related Factor 2
	NRG-1	Neuregulin-1
	NRGs	Neuregulins
	N^6-MB-cAMP	N^6-monobutyryl-cAMP
P	PAD	Peptidyl Arginine Deiminase
	PAF	Platelet-Activating Factor
	PAMPs	Pathogen Associated Molecular Patterns
	PBMC	Peripheral Blood Mononuclear Cell
	PBS	Phosphate Buffer Saline
	PCID	Phagocytosis-Induced Cell Death
	PDGFR	Platelet-Derived Growth Factor Receptor
	PE	Phycoerythrin
	PECAM	Platelet Endothelial Cell Adhesion Molecule
	PGP	Proline-Glycine-Proline
	PHDs	Prolyl Hydroxylase Domain-containing enzymes
	PI3K	Phosphoinositide-3-Kinase
	PKA	Protein Kinase A
	PKB	Protein Kinase B
	PKC	Protein Kinase C
	PKIS	Published Kinase Inhibitor Sets
	PMN	Polymorphonuclear Neutrophil
	PPL	Project Licence
	PPP	Platelet Poor Plasma
	PRP	Platelet Rich Plasma
	PS	Phosphatidyl Serine
	P-Selectin	Platelet Selectin
	PVDF	Polyvinylidene difluoride
	p^{VHL}	von Hippel-Lindau protein
Q	QVD	Q-VD-OPh hydrate
R	RA	Rheumatoid Arthritis
	RBC	Red Blood Cell

	RCF (or g)	Relative Centrifugal Force
	ROS	Reactive Oxygen Species
	RPM	Revolutions per minute
	rTEM	Reverse Transendothelial Migration
	RTKs	Receptor Tyrosine Kinases
S	SDS	Sodium Dodecyl Sulfate
	SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
	SEM	Standard Error of Mean
	SGK	serum and glucocorticoid-related kinase
	SSC	Side scatter
T	TBS	Tris-Buffered Saline
	TEM	Transendothelial Migration
	TEMED	Tetramethylethylenediamine
	TGF	Transforming Growth Factor
	Th1	Type 1 T helper cells
	Th17	IL-17-producing helper T cells
	TNF	Tumour Necrosis Factor
	TLRs	Toll-Like Receptors
	TRAIL	TNFα-Associated Apoptosis Inducing Ligand
	TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
V	VLA-1	Very Late Antigen-1

Chapter 1. Introduction

1.1. Neutrophils

The immune system is comprised of two arms: innate and adaptive immunity. The evolutionarily conserved innate immune system is the first line of defence that provides rapid and non-specific physical, chemical and microbiological barriers against a wide range of pathogenic organisms, and other noxious substances. The innate immune system encompasses cellular components including neutrophils, monocytes, macrophages, dendritic cells (DCs), natural-killer (NK) cells, mast cells, as well as complement, cytokines, and acute-phase proteins. On the other hand, adaptive immunity is highly specific, develops slowly, and is mainly mediated by T- and B-lymphocytes.

Neutrophils (also called polymorphonuclear neutrophils, PMNs) are a subdivision of myeloid-derived granulocytes. Granulocytes are so named because of the presence of a large number of cytoplasmic granules, and are comprised of neutrophils, eosinophils or basophils on the basis of their cytoplasmic staining and morphological features. Neutrophils represent the most abundant class of granulocytes (95%) and leukocytes (50-70%), and are descended from pluripotent hematopoietic stem cells (Baum et al., 1992, Becker et al., 1963, Cudkowicz et al., 1964). Under the influence of growth factors and cytokines in the micro-environment of bone marrow, pluripotent hematopoietic stem cells differentiate into myeloblasts (Killmann et al., 1962), which are developmental precursors of mature neutrophils. Neutrophils are formed in the bone marrow, and upon release they are capable of diapedesis into tissue. As a result of the high rate of emigration, circulating neutrophils are short-lived, with a half-life of 6-8 hours *in vivo* (Athens et al., 1961, Cronkite and Fliedner, 1964, Fliedner et al., 1964).

Neutrophils have long been considered as potent inflammatory immune cells that rapidly migrate to the site of injury or infection, and play an active role in eradicating bacterial and fungal pathogens via their phagocytic and microbicidal activities (Kaufmann, 2008). The importance of neutrophils in host-mediated innate immune responses is highlighted by the fact that patients with abnormally low levels of neutrophils (neutropenia) or defects in neutrophil function (Chronic Granulomatous Disease, CGD) are severely vulnerable to a wide range of microbial infections (Segal, 2005, Vento and Cainelli, 2003). In addition, recent research on neutrophil biology suggests that neutrophils have an important immune-modulatory role by cross-talking with other immune cells, and to regulate adaptive immune responses (detailed in **section 1.2.7**).

1.2. Neutrophils in host defence

In response to pathogens or foreign particles, neutrophils become activated by a series of complex signalling events that lead to a chemotactic response to inflamed/infected tissues. When there, they mobilise granules and secretory vesicles, undergo de novo gene expression to perform antimicrobial and immune-modulatory effector functions.

1.2.1. Recruitment of neutrophils to the site of inflammation or infection

To initiate an effective immune response against pathogenic organisms such as bacteria and fungi, mature neutrophils are recruited from the circulation or bone marrow reserves to the site of infection in a multistep process. Neutrophil recruitment is mediated by various host or pathogen-derived factors [e. g. IL-8 (Interleukin-8, CXCL8 [chemokine-CXC-ligand 8]), leukotriene B4 (LTB4), C5a, CXCL1, and CXCL5] which have chemotactic effects on neutrophils (Wengner et al., 2008). These chemotactic mediators are sensed by specific receptors expressed on the surface of

neutrophils and allow neutrophils to migrate towards inflammatory sites along the concentration gradient. The inflammatory chemokine IL-8, released from host cells such as macrophages, endothelial, epithelial and smooth muscle cells, is detected via CXC-chemokine receptor 1 (CXCR1) and CXCR2, and is a key neutrophil recruitment factor (Walz et al., 1987, Yoshimura et al., 1987). Pathogen-derived molecules, for example fMLP (N-Formyl methionyl-leucyl-phenylalanine) is detected via fMLP receptors (also called formyl peptide receptors, FPRs) and also has a direct chemotactic effect on neutrophil recruitment (Schmeling et al., 1979, Showell et al., 1976).

Neutrophil migration involves rolling, tethering, adhesion and subsequent traversing through the vascular endothelium into the tissue (**Figure 1.1**). Both the rolling and tethering processes involve a variety of glycoproteins called selectins (Lawrence and Springer, 1991). Neutrophils express CD162 (also called P-selectin glycoprotein ligand-1), which helps them to attach to the surface of endothelium by interacting with the endothelial P- and E-selectins, and these selectins facilitate the mechanism of rolling and tethering (Moore et al., 1995). Neutrophils also express β1- and β2-integrins (also called VLA-1 [Very Late Antigen-1] and LFA-1 [Lymphocyte function-associated antigen-1], respectively), which mediate the adhesion process (Ley, 2002, von Andrian et al., 1991), and once they complete firm adhesion, they eventually traverse through the endothelium. The migration of neutrophils through the endothelial barrier (called transendothelial migration, TEM) is proceed by two routes: paracellular route, which occurs through small gaps between endothelial cell junctions (Engelhardt and Wolburg, 2004, Hurley, 1963, Marchesi and Florey, 1960), and transcellular, which occurs through the endothelial cells themselves (Feng et al., 1998, Fernandez-Borja et al., 2010). The migration through the paracellular route is supported by the active movement of a number of endothelial cell junctional molecules, including platelet endothelial cell adhesion molecule-1 (PECAM-1),

intercellular adhesion molecule-2 (ICAM-2), endothelial cell-selective adhesion molecule (ESAM), CD47, activated leukocyte cell adhesion molecule-1 (ALCAM-1), CD99, and junctional adhesion molecules (JAMs) (Muller, 2015, Nourshargh and Alon, 2014, Nourshargh et al., 2010, Vestweber, 2015). Similar classes of molecules, such as PECAM-1, JAM-A and CD99, have been reported to support the transcellular migration (Carman et al., 2007, Mamdouh et al., 2009, Marmon et al., 2009, Nourshargh et al., 2010).

After an initial flux of neutrophil recruitment to the inflammatory site, neutrophil recruitment is further amplified in a phenomenon called ‘neutrophil swarming’ by which more neutrophils are recruited in response to the signals from the early recruited ‘pioneer’ neutrophils and other immune cells (such as macrophages) (Ng et al., 2011, Sadik and Luster, 2012). Neutrophil swarming ensures efficient immune mechanism by enriching their numbers at sites of inflammation and involves many of the same signals utilised in the early phases of neutrophil recruitment, such as IL-8 and LTB4 (Lammermann, 2015, Sadik and Luster, 2012).

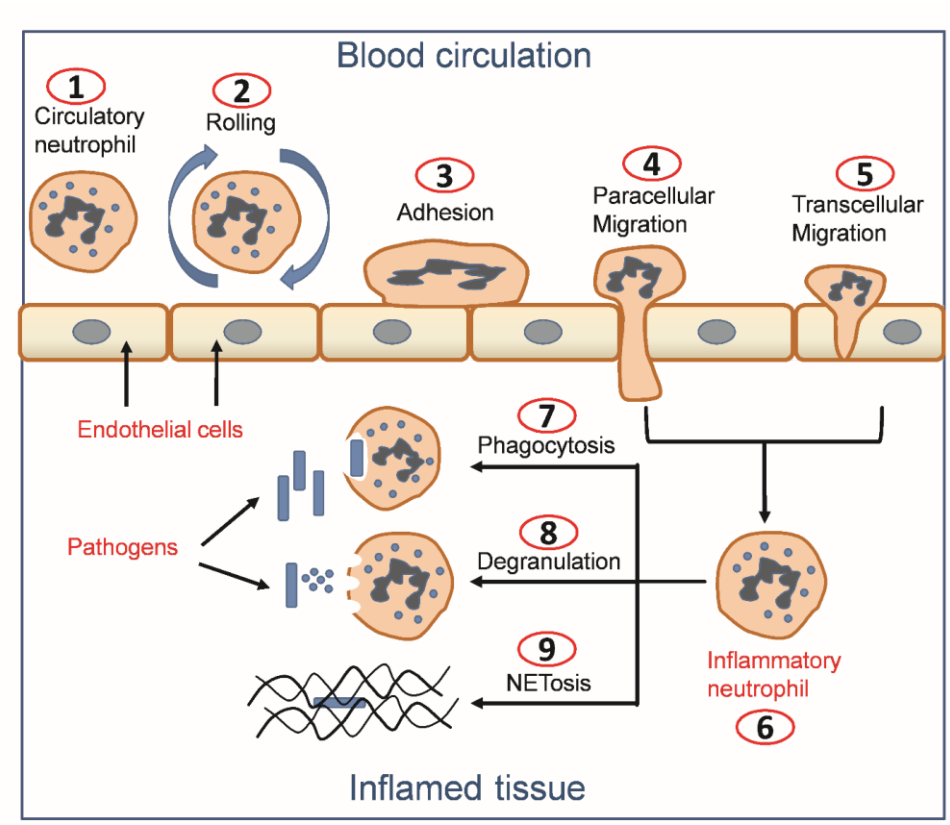


Figure 1.1. Neutrophil recruitment and immune effector functions. Circulatory neutrophils migrate to the inflammatory tissues by rolling and adhesion on the endothelium followed by traversing through the endothelium by either paracellular or transcellular migration towards the epicentre of inflammation, where they perform a number of immune functions such as phagocytosis, degranulation, and NETosis.

1.2.2. Priming

Neutrophil priming is an essential phenomenon in potentiating the cellular response to activating stimuli (Condliffe et al., 1998). Considerable evidence from *in vitro* studies suggest that neutrophils are primed by a variety of microbial (e.g. lipopolysaccharide [LPS] and platelet-activating factor [PAF]) (Guthrie et al., 1984, Vercellotti et al., 1988) and host (e. g. granulocyte-macrophage colony stimulating factor [GMCSF], tumour necrosis factor-α [TNF-α], IL-8, and Interferon- γ [IFN-γ]) factors (Berkow et al., 1987, Edwards et al., 1988, Ellis and Beaman, 2004, Mitchell

et al., 2003, Weisbart et al., 1986). A notable example is the priming effect of LPS on fMLP responses in neutrophils, where LPS priming induces the translocation and assembling the 'NADPH oxidase' (nicotinamide adenine dinucleotide phosphate-oxidase) components on the membrane and subsequent fMLP exposure results the rapid activation of the oxidase (El-Benna et al., 2008, Guthrie et al., 1984). Neutrophil priming is also induced by shearing or adhesion to artificial surfaces (Liles et al., 1995) and every process of the recruitment is a source of priming. Neutrophil priming enhances various functional consequences for the neutrophil such as enhanced integrin expression and release of superoxide (Condliffe et al., 1996), cell polarisation and degranulation (Condliffe et al., 1998, Fittschen et al., 1988). Since numerous neutrophil priming agents have been reported, a wide variety of pathways are thought to be involved in the priming processes. A number of notable pathways that are involved in neutrophil priming includes PI3K (phosphoinositide-3-kinase), ERK (extracellular signal regulated kinase) MAPK (mitogen activated protein kinase), p38 MAPK, PKA (protein kinase A) and PKC (protein kinase C) (Dang et al., 2001, Dang et al., 2003, Dewas et al., 2000, El-Benna et al., 2008, Kodama et al., 1999).

In contrast to the priming effects of activating stimuli, neutrophils are also reported to be desensitised to the effects of certain priming factors; for example, in cases of repeated stimulation by a chemoattractant, neutrophils often show desensitisation by down-regulating the chemoattractant-receptors by endocytosis (Claing et al., 2002, Didsbury et al., 1991).

1.2.3. Granules and degranulation

Neutrophils secrete a plethora of potentially dangerous substances onto pathogens as part of their microbicidal activities. Neutrophils have three types of classical granules (**Table 1.1**), which contain over 300 proteins involved in neutrophil adhesion, migration and anti-microbial activities. First, azurophilic (sometimes called primary or peroxidase positive) granules are the largest, are myeloperoxidase (MPO) positive

and their synthesis typically starts at the beginning of neutrophil maturation (Faurschou and Borregaard, 2003). MPO is essential for respiratory burst activity (Lacy, 2005, Nusse and Lindau, 1988), and is noted to play a key role in neutrophil activation (Lau et al., 2005), pathogen killing (Allen and Stephens, 2011, Hirche et al., 2005, Humphreys et al., 1989) and NET (Neutrophil Extracellular Trap) formation (Metzler et al., 2011). Further to the MPO, the azurophilic granule also contains defensins, “bactericidal permeability increasing protein” (BPI), lysozyme and aminopeptidases such as cathepsin G, neutrophil elastase (NE) and proteinase 3, all of them are known to have anti-microbial activities (Faurschou and Borregaard, 2003). The second set of granules are called secondary (or specific) granules, which contain a glycoprotein called lactoferrin, lysozyme and anti-microbial proteins such as ‘human cathelicidin antimicrobial protein’ (hCAP) -18, ‘neutrophil gelatinase-associated lipocalin’ (NGAL) but no myeloperoxidase, and are smaller than azurophilic granule and formed after the azurophilic granule (Faurschou and Borregaard, 2003, Lacy, 2005). The third set of granules (tertiary or gelatinase) are peroxidase negative, smaller than specific granules, contain metalloproteinases (e.g. gelatinase) and considered as the last granule formed in the maturation process (Borregaard, 2010, Faurschou and Borregaard, 2003). All three granule types emerge from the Golgi apparatus during the maturation stages of neutrophils. Finally, neutrophils also harbour another class of structures, called vesicles, which are produced by the process of endocytosis during the final stage of the maturation rather than budding from Golgi apparatus (Borregaard et al., 2007). These granules not only have a direct cytotoxic effect on microbes but also augment other functional activities of neutrophils; for example the granule-derived MPO augments microbicidal activity via NADPH oxidase-mediated ROS production (Klebanoff, 1968, Klebanoff, 1974, Rosen and Klebanoff, 1977, Rosen and Klebanoff, 1979, Schultz et al., 1965), pathogen killing (Allen and Stephens, 2011, Hirche et al., 2005, Humphreys et al., 1989) and NET formation (Metzler et al., 2011).

During the course of neutrophil activation, the granules are mobilised and fused with the plasma and phagosomal membrane leading to release of the anti-microbial components in order to create an inhospitable environment for pathogens (Faurschou and Borregaard, 2003). The propensities of mobilisation of different classes of granules vary: highest for secretory vesicles followed by gelatinase granules, specific granules and finally, azurophilic granules (Borregaard et al., 1992, Borregaard et al., 1994, Kjeldsen et al., 1993, Kjeldsen et al., 1992, Sengelov et al., 1993).

Table 1.1. Types of neutrophil granules and their important cytotoxic proteins[†].

Granule types		Primary (azurophilic)	Secondary (specific)	Tertiary (gelatinase)	Secretory vescicles
Granulation propensity		++	+++	++++	+++++
Characteristic Proteins					
	Lysozyme	+	+	+	
	Myloperoxidase	+			
	Lactoferrin		+		
	Elastase	+			
	Gelatinase			+	
	Defensin	+			
	Complement Receptor 1			+	+
	FCgRIII			+	+

[†] Table reproduced from (Amulic et al., 2012).

1.2.4. Reactive Oxygen Species (ROS) generation

As part of the antimicrobial killing mechanisms, activated neutrophils produce reactive oxygen species (ROS). The generation of ROS is mediated by the NADPH oxidase, a multicomponent enzyme system, located at the plasma or phagosomal membrane.

The process of generating ROS is often referred to as “respiratory burst” activity, in which NADPH oxidase reduces molecular oxygen to superoxide radicals, which are readily converted to hydrogen peroxide spontaneously or by superoxide dismutase (Klebanoff, 1968, Klebanoff, 1974). Hydrogen peroxide is subsequently metabolised by MPO to more potent reactive radicals such as hypochlorous acid, hydroxyl radicals, and singlet oxygen species (Klebanoff, 1968, Klebanoff, 1974, Rosen and Klebanoff, 1977, Rosen and Klebanoff, 1979). ROS have been reported to play role in diverse neutrophil functions such as pathogen killing (Klebanoff, 2005, Nauseef, 2007), NET formation (Bianchi et al., 2009, Fuchs et al., 2007) and cell signalling (Fialkow et al., 2007, Winterbourn et al., 2016). The importance of ROS in the neutrophilic innate immune defence is evident from a rare hereditary disease, CGD, characterized by a genetic defect in NADPH oxidase (NOX2) and these patients are frequently infected with bacterial and fungal pathogens (Hohn and Lehrer, 1975).

Neutrophils also produce reactive nitrogen species called ‘nitric oxide’ (NO) by primary granule enzyme, inducible nitric oxide synthase (iNOS), which is induced in response to priming factors (e.g. IFN-γ, IL-1 and TNF) (Evans et al., 1996) and during infection (Wheeler et al., 1997). NO was noted to complement the ROS production from the evidence of a murine study where mice lacking both the iNOS and NADPH oxidase develops spontaneous infections by commensal microbes whereas mice with a single gene knockout do not develop such infections (Shiloh et al., 1999). NO also participates in cell signalling (Fialkow et al., 2007).

Despite their importance in microbicidal activities, neutrophil-derived ROS (and granule proteases) also contribute to lung injury (Kawabata et al., 2000, Wang et al., 1994). This will be discussed further in **section 1.4.2.**

1.2.5. Phagocytosis

Phagocytosis is an active process of ingesting pathogens, or pathogen-derived molecules, leading to the formation of a phagosome where antimicrobial activities take place (Nordenfelt and Tapper, 2011). Initiation of phagocytosis begins by receptor-mediated recognition of pathogens or pathogen-derived molecules (e.g. LPS, peptidoglycan, double-stranded viral RNA etc.) or host factors (e.g. complement components, antibodies etc.). Neutrophils express Toll-like receptors (TLRs) on their surface that help them in the recognition of 'pathogen associated molecular patterns' (PAMPs) present on microbial surfaces and promote microbial recognition for phagocytosis. Phagocytosis is more effective if microbes are opsonised by complement or antibodies, which neutrophils recognise via complement receptors such as ClqR56, CD35 (CR1) (Rabellino et al., 1978, Ross et al., 1978), CD11b/CD18 (CR3) (Rabellino et al., 1978, Ross et al., 1978), and CD11c/CD18 (CR4) (Myones et al., 1988). In addition, they express receptors for CD23 (FcεRI, IgE receptor) (Gounni et al., 2001), CD89 (FcαR, IgA receptor) (Albrechtsen et al., 1988), CD64 (FcγRI, IgG receptor) (Mantovani, 1975), and CD16 (FcγRIIIb, low-affinity IgG receptor) (Mantovani, 1975) that recognize antibody-coated microbes and facilitate phagocytosis. Receptor-mediated recognition is followed by engulfment, which involves invagination of the cell membrane and internalisation of microbes into a closed phagosome. The mechanism of phagocytosis is not completely understood, however, the "zipper mechanism" is a well-accepted model of uptake mechanism, which describes the engulfment of particles occuring by a step-by-step engagement of the phagocyte membrane with the surface of the particle, in a zipper like fashion (Decuzzi and Ferrari, 2007, Herant et al., 2006, van Zon et al., 2009). According to the Zipper mechanism, the receptor-ligand interactions between the phagocyte and the pathogen triggers signalling cascades to remodel the actin cytoskeleton in order to physically internalise pathogens into a phagosome (Ham et al., 2011, Tollis et al., 2010). The phagosome undergoes maturation and becomes progressively more

inhospitable to the cargo (Flannagan et al., 2012, Nordenfelt and Tapper, 2011). Cytoplasmic granules fuse with the phagosome forming the phagolysosome, exposing the bacteria to cytotoxic peptides and proteases (Flannagan et al., 2012, Nordenfelt and Tapper, 2011). Concurrently, ROS generated by NADPH-oxidase located at the phagosomal membrane also creates a cytotoxic environment which aids the bacterial killing (Roos et al., 2003). Neutrophil phagocytosis differs from the phagocytosis by other phagocytes in several ways. For example, the phagosome maturation process is much slower in neutrophils (Vieira et al., 2002) and the pH of the neutrophil phagosome is higher than in other professional phagocytes such as macrophages (Jankowski et al., 2002).

Although neutrophils facilitate killing of microbes by creating a hostile environment within the phagosome, a number of pathogens develop strategies to either prevent this process or to survive within the phagosome. For example, *Staphylococcus aureus* interferes with the process of phagocytic engulfment via polysaccharide capsules (Luong and Lee, 2002). *Francisella tularensis* prevents NADPH oxidase-mediated ROS production (McCaffrey et al., 2010). Although *Helicobacter pylori* induces an oxidative burst, they divert the NADPH oxidase so that the superoxide anion accumulates in the extracellular space (Allen et al., 2005b). *Streptococcus pyogenes* and *Salmonella typhimurium* both interfere with the fusion of granules with the phagosome (Joiner et al., 1989, Staali et al., 2006).

1.2.6. NETs (Neutrophil Extracellular Traps)

In addition to phagocytic antimicrobial activities, neutrophils also perform their anti-microbial agenda using a mechanism called NETs. NETs are the result of a unique form of neutrophil death called NETosis, which is morphologically different from apoptosis and necrosis (Fuchs et al., 2007). NET formation begins with the loss of nuclear lobes, chromatin condensation, and the detachment of inner and outer

nuclear membranes. In parallel, the cytoplasmic granules disintegrate. Finally, the cell starts to contract followed by loss of the cytoplasmic membrane and subsequent release of internal contents into extracellular space (Brinkmann et al., 2004, Fuchs et al., 2007). The condensed chromatin, in association with the granule and cytoplasmic proteins, forms extracellular fiber-like structures which trap and kill extracellular microorganisms (Urban et al., 2009, Brinkmann et al., 2004, Urban et al., 2006). A number of inducers of NET formation have been reported, ranging from pathogens such as bacteria (Brinkmann et al., 2004), fungi (Raftery et al., 2014, Urban et al., 2006) and viruses (Urban et al., 2006) to host factors such as activated endothelial cells (Gupta et al., 2010), IL-8 (Gupta et al., 2005, Gupta et al., 2010), ROS (Bianchi et al., 2009, Fuchs et al., 2007), NE (Papayannopoulos et al., 2010), MPO (Metzler et al., 2011) and G-CSF (granulocyte colony stimulating factor) (Demers et al., 2012). The importance of ROS in NET formation is evident from the fact that CGD patients with defects in NADPH oxidase cannot form NETs, which may impact on their susceptibility to infection (Bianchi et al., 2009, Fuchs et al., 2007). NE and MPO, have also been reported to be involved in the formation of NETs. Neutrophils from donors who have severe MPO deficiency fail to undergo NET formation suggesting MPO is essential in this process (Metzler et al., 2011). Furthermore, NE-deficient mice failed to form NETs and had impaired immune function (Papayannopoulos et al., 2010). A number of signalling pathways such as Rho-MEK (mitogen activated kinase/ERK kinase)-ERK (Hakkim et al., 2011), autophagy (Remijsen et al., 2011) and PAD (peptidyl arginine deiminase) (Wang et al., 2009), have been reported to facilitate NET formation. Although NET formation was initially described as an anti-microbial mechanism, because of the collateral damage caused by NETs, excessive or dysregulated NET formation can damage healthy host tissues and may lead to a number of pathologies, including inflammatory diseases such as rheumatoid arthritis (RA) and lung injury (Caudrillier et al., 2012, Caudrillier and Looney, 2013, Saffarzadeh et al., 2012, Thomas et al., 2012, Xu et al., 2009).

1.2.7. Interaction of neutrophils with other Immune Cells

Neutrophils not only perform their own anti-microbial activities, but they also interact with other immune cells by producing cytokines or chemokines and thus regulate both innate and adaptive immune responses (Amulic et al., 2012, Mantovani et al., 2011, Mocsai, 2013). Tissue neutrophils directly recruit neutrophils by releasing IL-8 in a paracrine manner (Scapini et al., 2000) and also indirectly by generating pro-inflammatory cytokines (e.g. TNFα, IL-1β) which act on other cells to produce neutrophil chemoattractants (Kasama et al., 2005, Sica et al., 1990).

Neutrophils express and produce chemokines such as (monocyte chemoattractant protein-1) MCP-1, macrophage inflammatory protein-1α (MIP-1α, also called CCL3), MIP-2α that are considered as classical chemoattractants for monocytes (Katsura et al., 1993, Scapini et al., 2001, Yoshimura and Takahashi, 2007). In addition, monocyte- and macrophage-mediated cytokine production and phagocytosis are also modulated by neutrophil-derived granule proteins (Soehnlein et al., 2008, Soehnlein et al., 2009).

Neutrophils also play an important role in the recruitment and activation of dendritic cells (DCs). DCs are potent antigen presenting cells that activate T-cell mediated adaptive immune responses against invading pathogens (Mellman and Steinman, 2001). Neutrophils secrete the chemokine, CCL3, that recruits DCs to the site of infection (Charmoy et al., 2010) and also induce DCs to secrete IL-12, which has a proliferative effect on T-cells (Bennouna et al., 2003, van Gisbergen et al., 2005), thus regulating the adaptive immune responses.

Recent research suggests that neutrophils, NK cells and DCs interact with each other via cytokines as well as by direct cell-cell contact, and provide an important foundation for immune responses (Costantini, 2011, Sporri et al., 2008). NK cells are innate lymphocytes which participate in anti-viral and anti-tumour immune defence. In

particular, both IL-18 (released from neutrophils) and IL-12 (released from DCs) induce NK cells to produce IFN-γ (Sporri et al., 2008). There is a proposal that a trio complex, consisting of neutrophils, DCs and NK cells, forms at inflammatory sites (including Crohn's disease and psoriasis) and the complex helps in the production of IFN-γ by NK cells (Costantini, 2011).

In addition to innate immune cells, neutrophils also communicate with adaptive immune cells such as T- and B-cells. Neutrophil derived IL-12 was implicated in Th1 (Type 1 T helper) cell differentiation (Romani et al., 1997, Tateda et al., 2001). Furthermore, neutrophils also express a number T-cell chemoattractants and factors that promote B-cell differentiation (Huard et al., 2008, Scapini et al., 2008, Scapini et al., 2000). Th1-cell derived IFN-γ is also noted to enhance neutrophil lifespan and phagocytosis (Ellis and Beaman, 2004). Th17 (IL-17-producing helper T cells) cells secrete IL-17 which can modulate neutrophil phenotypes indirectly via increased expression of G-CSF, IL-8 and TNF-α by endothelial and epithelial cells (Ouyang et al., 2008).

1.3. The resolution of neutrophilic inflammation

The cytotoxic activities of neutrophils not only act against pathogens but may also pose detrimental effects to host tissues when inappropriately activated. Therefore, the release of cytotoxic substances by neutrophils must be well regulated so that they do not cause detrimental harm to the host. There are important cellular regulatory events that allow the host to limit the neutrophil mediated inflammatory response (Hallett et al., 2008, Leitch et al., 2008). One of the important regulatory mechanisms in inflammation resolution is neutrophil apoptosis.

1.3.1. Neutrophil apoptosis

Apoptosis (programmed cell death) is an active cellular process required to maintain tissue homeostasis. The short-lived neutrophil dies by constitutive apoptosis (Savill et al., 1989b, Whyte et al., 1999), where the cell membrane remains intact to limit the loss of cytotoxic contents, and then cleared by macrophages in a process known as efferocytosis (Savill et al., 1989b, Whyte et al., 1999, Whyte et al., 1993, Savill et al., 1989a). Inhibition of apoptosis and/or efferocytosis can interfere with inflammation resolution, ultimately contributing to tissue damage (**Figure 1.2**) (Rossi et al., 2006, Savill and Haslett, 1995), and is observed in many inflammatory conditions such as chronic obstructive pulmonary disease (COPD), rheumatoid arthritis (RA) and inflammatory bowel disease (IBL) (Gilroy et al., 2004, Serhan and Savill, 2005, Taylor and Colgan, 2007).

Apoptotic neutrophils characteristically feature chromatin condensation, nuclear fragmentation, cell shrinkage, and phosphatidyl serine (PS) exposure on the surface of the plasma membrane (Martin et al., 1995, Savill et al., 1989b). On the contrary, necrotic cell death is distinct from the apoptosis process and is characterised by loss of membrane integrity, increased cell volume and untimately cell lysis (Wyllie et al., 1980).

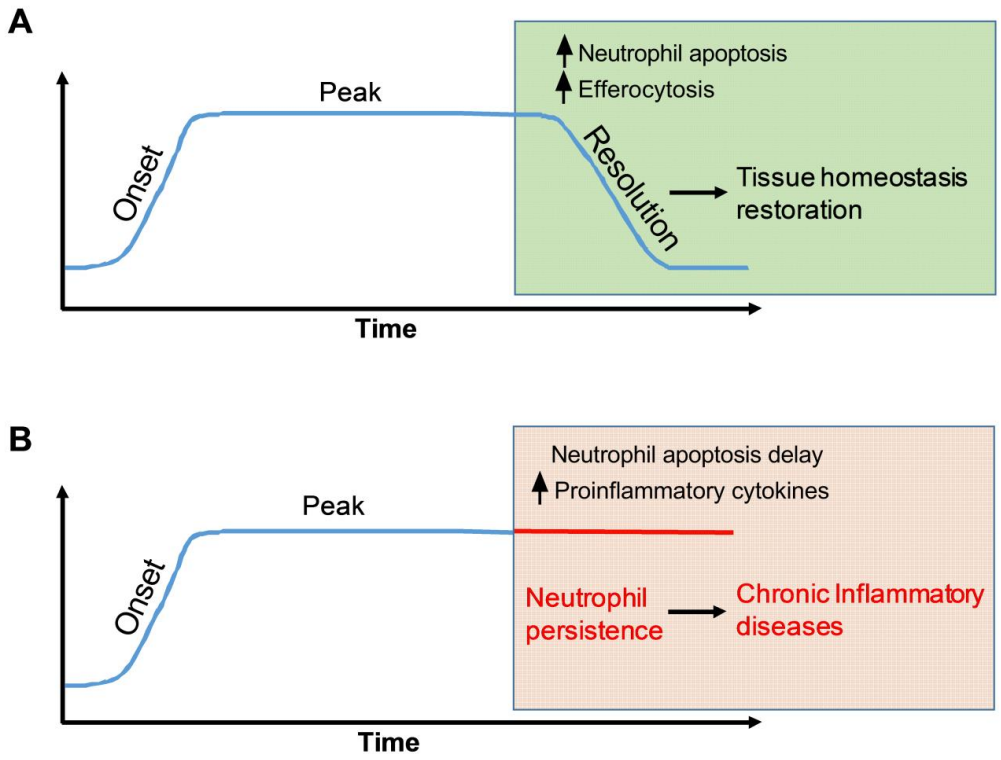


Figure 1.2. Schematic diagram of neutrophilic inflammation. As a key immune cell in an inflammatory response, neutrophil rapidly recruited to and enriched in the sites of inflammation to perform their immune functions. To maintain physiological balance, neutrophils undergo neutrophil apoptosis followed by macrophage mediated efferocytosis clearance, restoring the neutrophil numbers at homeostatic levels (panel A). However, delayed neutrophil apoptosis may also result the persistence of neutrophils at inflamed tissues contributing to chronic inflammation (panel B). The figure was adapted from (Serhan et al., 2007).

1.3.1.1. Apoptotic and anti-apoptotic signals

The inflammatory microenvironment regulates neutrophil apoptosis (Luo and Loison, 2008, Simon, 2003). Prolonging neutrophil lifespan enables neutrophils to efficiently combat against infection, but if dysregulated, can also perpetuate inflammation. A number of pathogen-derived factors (e.g. LPS, peptidoglycan and CpG DNA) promote neutrophil survival via TLR activation (Colotta et al., 1992, Francois et al., 2005). Pro-inflammatory cytokines and growth factors such as GMCSF, G-CSF, IFN-γ and IL-1β are important neutrophil survival stimuli, whereas Fas ligand, TNF-α, and

TRAIL (TNFα-associated apoptosis inducing ligand) promotes apoptosis (Colotta et al., 1992, El Kebir et al., 2007, Gilroy et al., 2004, Khreiss et al., 2002, Lee et al., 1993, Renshaw et al., 2003). Pathogens subvert neutrophil apoptosis for their own gain, such as *Pseudomonas aeruginosa* which produces the pro-apoptotic toxin Pyocyanin (Usher et al., 2002). In addition to these, hypoxia (low oxygen pressure in tissue) is also a key neutrophil survival stimulus (Elks et al., 2011, Walmsley et al., 2005) (will be discussed detail in **section 1.5**)

1.3.1.2. Pathways of neutrophil apoptosis

Like nearly all cell types, neutrophil apoptosis involves two complex signalling pathways: intrinsic and extrinsic (**Figure 1.3**). The intrinsic pathway mediates apoptosis through the disruption of mitochondrial transmembrane potential leading to release of cytochrome C and endonuclease G (Luo and Loison, 2008, Simon, 2003), however, exactly what triggers induces these events is still obscure. Cytochrome C, a pro-apoptotic factor, oligomerises Apaf-1 (Apoptotic protease activating factor-1) triggering Caspase-9 and Caspase-3 activation (Green, 2000). The mitochondrial membrane integrity is controlled by a number of Bcl-2 (B-cell lymphoma-2) family proteins which include both pro-apoptotic (BAX [Bcl-2-associated X protein], BAK [Bcl-2 homologous antagonist/killer]) and anti-apoptotic (Mcl-1, Bcl-xL, A1) members (Bianchi et al., 2006, Witko-Sarsat et al., 2011). BAX and BAK insert into the mitochondrial membrane and promote the disruption of the mitochondrial membrane and this process is inhibited when they are complexed with anti-apoptotic Bcl-2 family proteins (Akgul et al., 2001, El Kebir et al., 2007). Mature neutrophils are noted to express lower levels of the anti-apoptotic proteins compared to the precursors of neutrophils (Geering and Simon, 2011

, Theilgaard-Monch et al., 2005), implying the importance of this pathway in constitutive neutrophil apoptosis. The extrinsic pathway involves the conveying of death signals through TRAIL, TNF-α or Fas death receptors followed by DISC (Death

Inducing Signalling Complex) formation and activation of adaptor proteins such as Fas-associated death domain (FADD) leading to cleavage of caspase-8 (Green, 2000). Fas signalling however, is thought to have little or no role in constitutive neutrophil apoptosis (Scaffidi et al., 1998, Scheel-Toellner et al., 2004, Schwartz et al., 2012, Serrao et al., 2001). While there is evidence of caspase-8 activation in constitutive apoptosis, the mechanism of the activation is independent of death receptors (Fecho and Cohen, 1998, Scheel-Toellner et al., 2004). In contrast, death receptor mediated neutrophil apoptosis plays a pivotal role at sites of inflammation (Jonsson et al., 2005, Nadeau et al., 2008).

ROS can activate the intrinsic (Arruda et al., 2006) and extrinsic (in a mechanism independent of death receptor mediated activation) (Scheel-Toellner et al., 2004) pathways of apoptosis. ROS damages DNA and activates the p53 pathway (Ye et al., 1999). The participation of ROS in promoting of constitutive neutrophil apoptosis came from anti-oxidant studies and patients with chronic granulomatous disease (Kasahara et al., 1997, Segal, 2005, Vento and Cainelli, 2003).

Further to the extrinsic and intrinsic pathway of neutrophil apoptosis, phagocytosis-induced cell death (PICD) also play an important role at the sites of infection and is initiated by microbial killing (Kennedy and DeLeo, 2009, Kobayashi et al., 2003, Kobayashi et al., 2002). It has been noted that NADPH oxidase derived ROS triggers the PCID in neutrophils (DeLeo, 2004, Kobayashi et al., 2003, Kobayashi and Deleo, 2004).

A number of partially overlapping signalling pathways are activated in neutrophil survival, including MAPK/ERK, PI3K/AKT (protein kinase B) and NF-κB (nuclear factor-kappa B), and their activation is mainly triggered by the agonism of growth factor receptors and TLRs (El Kebir and Filep, 2013a, Geering et al., 2013, Kennedy

and DeLeo, 2009). In this context, the prosurvival effects of LPS, GMCSF and G-CSF have been extensively studied and noted to act via PI3K/AKT (Rane and Klein, 2009) and MAPK/ERK signalling (Klein et al., 2000, Suzuki et al., 1999, Sweeney et al., 1999).

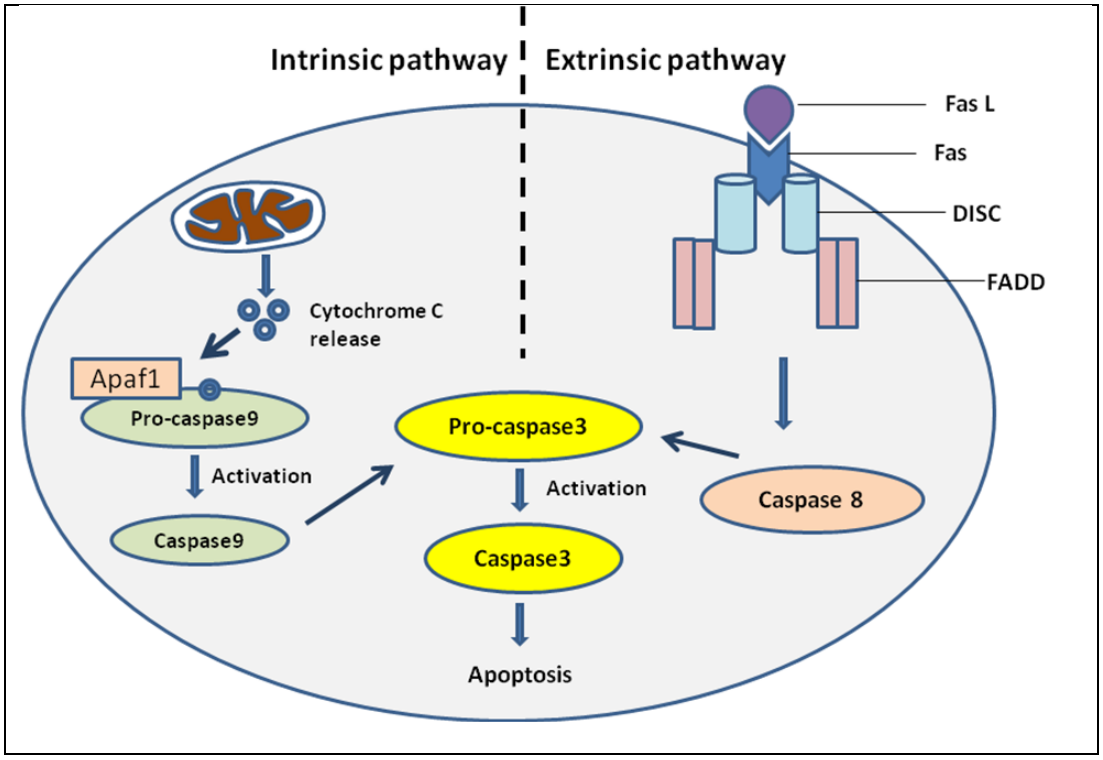


Figure 1.3. Intrinsic and extrinsic pathways of neutrophil apoptosis. The Intrinsic pathway initiates by the disruption of mitochondrial transmembrane potential due to ROS production and results in cytochrome C release, which subsequently activates caspase -9 and -3 activation leading to apoptosis. On the contrary, extrinsic pathway initiates by binding of Fas L with Fas receptor followed by formation of DISC and FADD that signals caspase-8 and -3 activation. [This figure was reproduced from (Favaloro et al., 2012)].

1.3.1.3. Protein kinases and neutrophil apoptosis

Protein kinases are an evolutionary conserved important class of proteins that phosphorylate target proteins and play intricate roles in signal transduction pathways

involved in numerous cellular processes, including cell proliferation, differentiation, and apoptosis (Hubbard and Cohen, 1993). Despite their importance in normal physiologic functions, kinases are implicated in a range of human diseases when aberrantly activated (Lahiry et al., 2010). The human kinome consists of 518 annotated kinases (Manning et al., 2002) and kinase inhibitors are increasingly considered as an important class of therapeutics for a growing range of human diseases (Singh et al., 2016, Wu et al., 2015).

A number of kinase signalling pathways are involved in neutrophil survival or apoptosis responses, and thus determine the fate of neutrophils. Pro-inflammatory mediators activate PI3K or MAPK/ ERK pathways that target and inhibit the intrinsic pathway of apoptosis (Luo and Loison, 2008, Simon, 2003). PI3K phosphorylates phosphoinositol derivatives, which results in activation of AKT and NF-kB pathways thus generating survival signals. Moreover, AKT and ERK phosphorylate BAD (Bcl-2-associated death promoter) and BAX, pro-death members of the Bcl-2 protein family, leading to dissociation of BAD and BAX from an anti-apoptotic protein Mcl-1 (Akgul et al., 2001, El Kebir et al., 2007). Again, inhibition of neutrophil apoptosis is associated with p38-MAPK, which provides survival signals by phosphorylation of caspase-8 and caspase-3 (Alvarado-Kristensson et al., 2004). In addition to all of these kinases, serum and glucocorticoid-related kinase 1 (SGK1) (Burgon et al., 2014, Klein et al., 2000) and cyclin-dependent kinases (CDKs) (Dorward et al., 2017, Hoodless et al., 2016, Leitch et al., 2010, Rossi et al., 2006), have been noted to regulate neutrophil apoptosis pathways. A number of studies showed that inhibition of kinases (such as CDKs, and SGK1) are implicated in promoting neutrophil apoptosis leading to inflammation resolution (Burgon et al., 2014, Dorward et al., 2017, Leitch et al., 2010, Rossi et al., 2006, Wang et al., 2003), suggesting a potential opportunity of targeting these and other potential kinases in designing effective drug for chronic inflammatory diseases. The key kinase regulators in neutrophil survival

pathways, which can potentially be therapeutically targeted to promote neutrophil apoptosis, are shown in **Figure 1.4.**

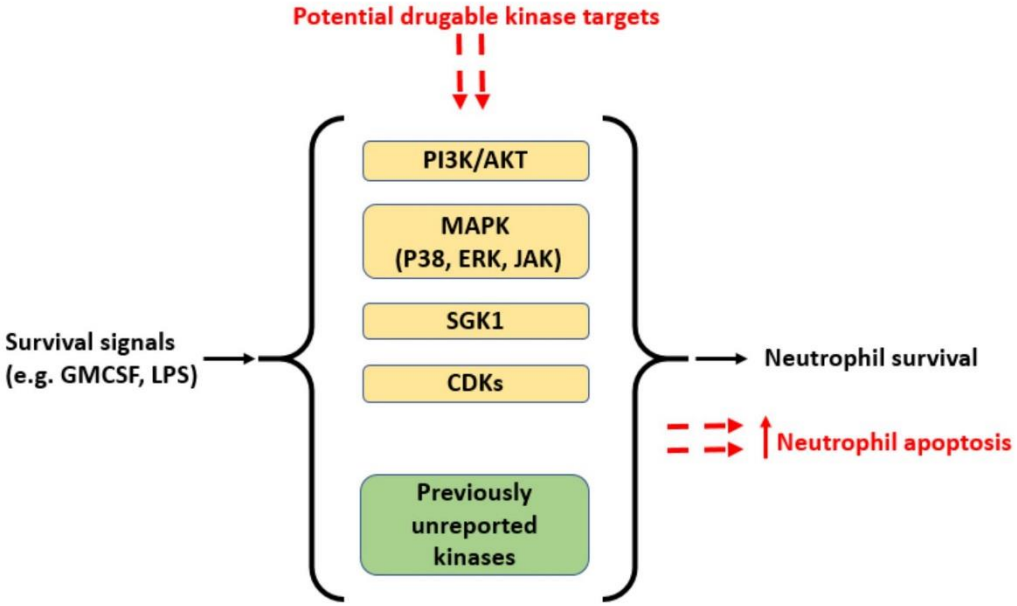


Figure 1.4. **Potential drugable kinase targets to promote neutrophil apoptosis**. In response to survival stimuli, a number of kinase signalling, including PI3K/AKT and MAPKs [p38, ERK, JAK (Janus Kinase)], were reported to be activated in neutrophils leading to the generation of survival response. In addition to these, SGK1 and CDKs were noted to regulate neutrophil survival. These and other kinases with previously unreported roles in neutrophil survival could be exploited as potential drugable targets for chronic inflammatory diseases.

1.3.2. Retrograde chemotaxis as a novel resolution mechanism

Retrograde chemotaxis or reverse migration is a well-established phenomenon by which immune cells such as lymphocytes and monocytes, migrate back to the circulation from the site of inflammation (Bradfield et al., 2007, McGettrick et al., 2009, Randolph et al., 1998), enabling them to be potentially re-used. It has since been shown in *in vivo* zebrafish models that neutrophils also migrate back through the endothelium towards the vasculature (known as reverse transendothelial migration,

rTEM), as part of a novel anti-inflammatory mechanism (Mathias et al., 2006, Starnes and Huttenlocher, 2012). Neutrophil reverse migration has not only been studied in models in zebrafish (Elks et al., 2011, Ellett et al., 2015, Robertson et al., 2014, Tauzin et al., 2014) but also in multiple models in mice (Duffy et al., 2012, Woodfin et al., 2011) and *in vitro* human neutrophils (Hamza and Irimia, 2015, Hamza et al., 2014).

Although the mechanism of neutrophil reverse migration is not well understood, studies have identified the involvement of a number of pathways. HIF (hypoxia-inducible factor)-1α has been implicated in the regulation of reverse migration, since the activation of this has been reported to enhance the retention of neutrophil at inflamed sites in a zebrafish model (Elks et al., 2011). Furthermore, neutrophil chemoattractants (e.g. IL-8) was described as chemorepellents for a subset of neutrophils at high concentration (Boneschansker et al., 2014, Tharp et al., 2006), thus it is possible that neutrophil behaviour can be changed during the course of migration along the concentration gradient of chemoattractants and this changes may play an important role in the homeostatic control of inflammation. Although these studies described the observation as a chemorepulsive effect, however, it is expected neutrophil migration to be stopped as a result of complete saturation of the IL-8 receptors rather than reverse migration. Therefore, the reverse migration phenomenon in response to IL-8 could potentially be governed by other unknown mechanism. Furthermore, these studies showed that 1.2 μM IL-8 had chemorepulsive effects compared to 120 nM IL-8, and the presence of this high concentration of IL-8 is very unlikely in actual physiological condition. Therefore, the chemorepulsive effects may not exist under physiological or pathophysiological contexts. In addition to this, reverse migrated neutrophils have been demonstrated to reduce the expression of CXCR1 (Buckley et al., 2006), perhaps suggesting that neutrophils may also show lower sensitivity to chemoattractants at the site of inflammation. It is also further demonstrated that the pro-resolution lipid mediator, lipoxin A4 (LXA4) can

enhance reverse migration of human neutrophils *in vitro* (Hamza et al., 2014). In a murine model of inflammation after ischemia-reperfusion injury, the rTEM phenomenon was observed (Colom et al., 2015, Woodfin et al., 2011). In this model system, it has been reported that at endothelial junctions a molecule called 'junctional adhesion molecule-C (JAM-C) was reported to modulate rTEM by decreasing its level and function after injury. In addition to this, the neutrophil chemoattractant, LTB4 also induces the production of NE, which can cleave JAM-C and therefore results in the enhancement of rTEM.

1.4. Neutrophil persistence in chronic inflammation

If inflammation is unresolved due to any impairment of neutrophil apoptosis and/or in the process of their subsequent clearance by macrophages, this may lead to chronic inflammatory conditions which include COPD.

1.4.1. COPD

COPD is a worldwide health concern that has an increasing prevalence and mortality (Lozano et al., 2012). This obstructive pulmonary disease is characterised by an impairment of air flow which is not fully reversible and is the consequence of unresolved inflammation in lung tissues initiated by harmful particles or gases such as cigarette smoke or fossil fuels (Diette et al., 2012, Kurmi et al., 2010, Laniado-Laborin, 2009). Inflammation continues even after the exposure ceases, with an ongoing decline of lung function and often death. The symptoms of COPD include excess sputum production, shortness of breath and coughing (Lozano et al., 2012), and this disease may also cause some extra-pulmonary effects (Cockayne et al., 2012, Nussbaumer-Ochsner and Rabe, 2011). The inflammatory response and subsequent tissue damage involves inappropriate persistence of neutrophils,

macrophages and other inflammatory cells that cause cytotoxic effects to the relevant tissues (Brusselle et al., 2011).

COPD patients have comparatively greater numbers of neutrophils in their sputum and bronchoalveolar lavage (BAL) than in the lung parenchyma, suggesting that neutrophils migrate rapidly through the parenchyma to the airways (Keatings et al., 1996). Smoking is a key etiologic factor in COPD pathogenicity and smokers have a comparatively higher number of neutrophils in the parenchyma and the lamina propria compared to non-smoker controls, and the neutrophils are mostly distributed in small airways rather than large airways (Battaglia et al., 2007, Pesci et al., 1998). The role of neutrophils in disease progression is also suggested by the fact that COPD patients were found to have increased neutrophils in the smooth muscle of peripheral airways (the site of early disease in COPD) compared to healthy controls (Baraldo et al., 2004). The increased neutrophilia in lung tissue, in association with other inflammatory cells such as macrophages and CD8+ T-cells, provokes uncontrolled inflammatory responses which have detrimental effects on tissue damage. Neutrophils collected from sputum of COPD patients have increased NF-kB activity (Brown et al., 2009), consistent with the increased level of neutrophils in lung tissue being in part a result of an NF-kB-mediated survival effect. In addition to local neutrophilic activation in the lung, COPD is also associated with systemic activation of neutrophils, which may contribute to muscle wasting and exacerbate cardiovascular disease either directly or indirectly through increased production of C-reactive proteins (CRP) from the liver (Cockayne et al., 2012, Nussbaumer-Ochsner and Rabe, 2011).

A devastating feature in COPD is the recurrent episodes of the deterioration of lung functions referred to as ‘exacerbations’, which are frequently triggered by bacterial and viral infection (Sethi, 2011) and are associated with a decline in lung function, hospitalisation and often death (Donaldson et al., 2002). Bacterial colonisation in the airway is reported in stable COPD patients (Murphy et al., 2004, Parameswaran et

al., 2009), some of them exhibiting persistent colonisation with pathogenic bacteria, and those individuals not only demonstrated enhanced airway inflammation but also had increased exacerbation frequency and deterioration of lung function (Banerjee et al., 2004, Hill et al., 2000, Patel et al., 2002, Wilkinson et al., 2003).

A number of studies suggest that the innate immune functions of neutrophil are altered in COPD. Neutrophil phagocytosis was noted to be reduced in COPD (Fietta et al., 1988, Prieto et al., 2001), although the effect was not replicated in other studies (Muns et al., 1995, Walton, 2014). Neutrophils from COPD patients also demonstrate not only increased chemotaxis speed (Burnett et al., 1987, Milara et al., 2012, Sapey et al., 2011, Woolhouse et al., 2005) but also an impairment in the migratory accuracy (Sapey et al., 2011). Furthermore, COPD circulatory neutrophils have increased NET production *in vitro* (Sapey et al., 2011). Sputum neutrophils were also noted to increase NET formation in COPD both in stable and exacerbated conditions (Grabcanovic-Musija et al., 2015, Pedersen et al., 2015), and this correlated with disease severity (Dicker et al., 2017, Grabcanovic-Musija et al., 2015).

In addition to dysregulated chemotaxis and NET formation, a body of literature implies that neutrophil apoptosis is also imbalanced in COPD. Sputum (Brown et al., 2009) and circulatory neutrophil apoptosis was noted to be delayed in stable COPD patients (Zhang et al., 2012) and during exacerbations (Pletz et al., 2004, Schmidt-Ioanas et al., 2006). A different study, however, showed circulatory neutrophil apoptosis to be unchanged in stable COPD patients but reported the increased expression of surface markers (e.g. CD62L and CD11B) suggesting that neutrophils may be activated in other ways (Noguera et al., 2004). The study by Noguera et al could be limited by lower sample size (14 patients) compared to much higher number of sample sizes used in other studies showing the resistance to neutrophil apoptosis in COPD (47, 36 and 62 patients studied in Zhang et al, Pletz et al and Schmidt-Ioanas et al, respectively). Furthermore, the difference between these studies could also be

attributed by different neutrophil isolation methods used in these studies and also by factors such as pathogen colonization, any chronic hepatic, pulmonary, cardiovascular, renal or rheumatological conditions, diabetes mellitus, and the confounding effects of drugs such as corticoids and differing exposure to cigarette smoke, age of the subjects may have an impact on the result. Circulatory pro-survival cytokines are elevated in stable COPD (Biffl et al., 1996, Moermans et al., 2011) and further elevated during exacerbations (Wedzicha et al., 2000), which may have an impact on neutrophil survival and activation. Taken together, it has been suggested that neutrophil apoptosis is dysregulated either as a result of an inherent apoptosis defect in neutrophils or stimulation of prosurvival effects by increased levels of pro-inflammatory cytokines.

1.4.2. Consequences of the presence of neutrophils in the lung in COPD

Lung neutrophilia leads to tissue damage in a number of ways. ROS and proteinases cause direct damage to healthy tissues. NE activates macrophages to release LTB4, a pro-inflammatory cytokine (Hubbard et al., 1991) and degrades the elastin of lung parenchyma, resulting in the loss of elasticity. A key mediator in COPD is leukotriene A4 hydrolase (LTA4H), which is a pro-inflammatory enzyme with bi-functional activities: as a hydrolase and an aminopeptidase. The hydrolase activity of the enzyme converts LTA4 to LTB4 which is a pro-inflammatory agent and neutrophil chemoattractant. The aminopeptidase activity cleaves proline-glycine-proline (PGP) tripeptide, which is also a potent chemoattractant. Cigarette smoke inhibits the aminopeptidase activity thus increasing both PGP and LTB4 that attract further neutrophils to the site of inflammation leading to subsequent tissue damage and further lung malfunction (Snelgrove et al., 2010).

1.4.3. Oxidative stress and COPD

Considerable evidence suggests a link between oxidative stress and the progression of COPD (Cantin and Crystal, 1985). Oxidative stress is defined as an increased level

of oxidant burden and/or decreased amount of the endogenous anti-oxidant pool. The increased oxidative stress in patients with COPD is the result of increased exposure to noxious particles (e.g. cigarette smoke) or increased generation of endogenous ROS from various inflammatory and epithelial cells in the lung (Loukides et al., 2011, Rahman, 2005). Oxidative stress in COPD may also be the result of a decreased concentration of endogenous antioxidants as a result of decreased activity of the genes involved in anti-oxidant defence systems. Many of these genes are regulated by nuclear factor erythroid-2–related factor 2 (Nrf2), a master regulator of anti-oxidant genes (Cho et al., 2006) (**Figure 1.5**). Under normal conditions, Nrf2 in the cytoplasm is associated with a cysteine-rich protein, Keap1 and is targeted for proteasomal degradation. Under conditions of oxidative stress, oxidants oxidize the cysteine residue of Keap1, thus dissociating it from Nrf2 so that Nrf2 remains stable. The stable Nrf2 is then translocated to the nucleus to induce expression of more than 200 anti-oxidant enzymes and detoxifying enzyme genes, including heme oxygenase-1 (HO-1), glutathione peroxidase, glutathione-S-transferase, glutathione reductase, γ-glutamyl cysteine synthetase, thioredoxin, and catalase (Kobayashi and Yamamoto, 2006). Oxidative stress has been implicated in multiple events in lung pathology such as oxidative inactivation of anti-proteases and surfactants, membrane lipid peroxidation, mucus hypersecretion, mitochondrial respiration, alveolar epithelial injury, remodelling of the extracellular matrix, and apoptosis (Avery, 2011, Ciccia and Elledge, 2010). Cigarette smoke is one of the predisposing factors that play a role in the pathogenesis of COPD. Cigarette smoke contains thousands of compounds including reactive oxygen species which have the potential to provoke oxidative stress and has been implicated in the development of COPD (MacNee, 2001). The oxidants from cigarette smoke directly target and damage components of lung matrix (e.g. elastin and collagen) (Cantin and Crystal, 1985). Furthermore, cigarette smoke inhibits elastin synthesis and repair (Laurent et al., 1983), leading to the development of emphysema.

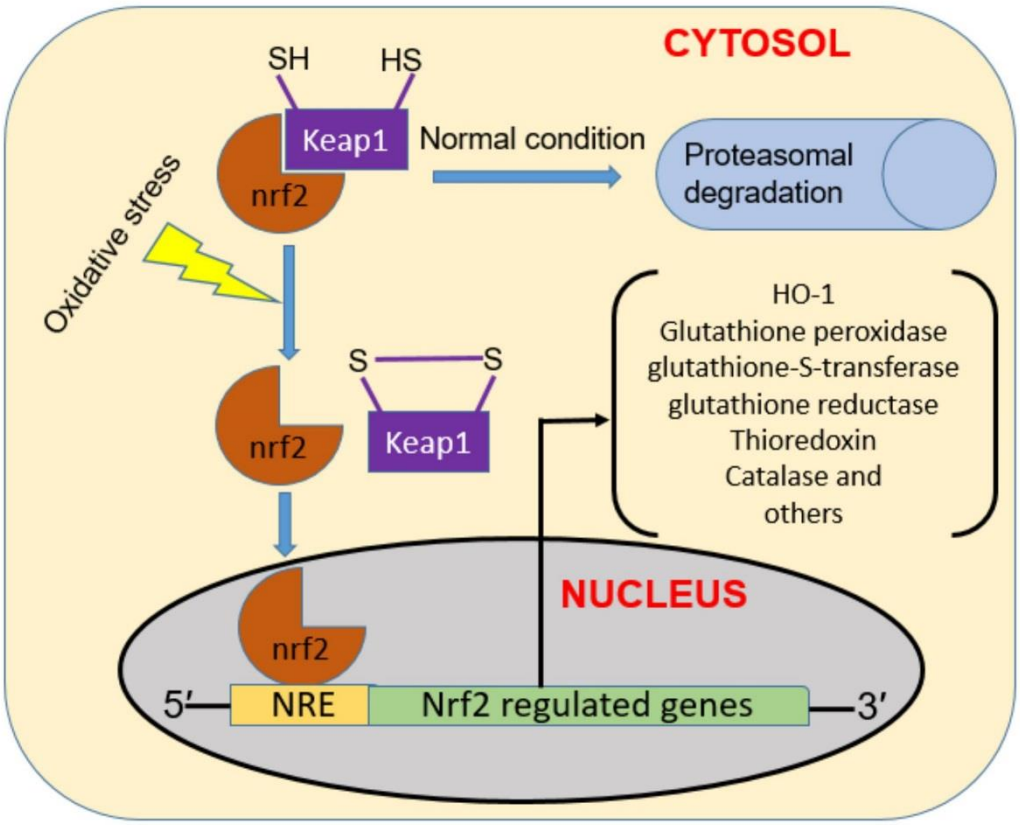


Figure 1.5. Oxidative stress-induced regulation of Nrf2. Under normal condition, Nrf2 is associated with a cysteine-rich protein Keap1 in the cytoplasm and is targeted for proteasomal degradation. In oxidative stress, the Keap1 is oxidised at cysteine residues, which results in the dissociation of Nrf2 from Keap1. Subsequently, Nrf2 after being translocated to nucleus binds with Nrf2 responsive elements (NRE) leading to switching on transcription of a large number of anti-oxidant genes. The figure was inspired from (Hellweg et al., 2016).

1.5. Neutrophils and hypoxia

The microenvironmental conditions in inflammatory tissues are characterized by hypoxia (low oxygen level in tissue) and low concentrations of other metabolites. During evolution, vertebrates have developed an oxygen compensation mechanism for their survival in low oxygen. This compensation mechanism has long been recognised from understanding the phenomena of cardiovascular adaptation and

erythropoiesis (Thompson et al., 2013). Cells respond to hypoxia by activating a nuclear transcription factor called HIF that helps the cells to adapt to low oxygen conditions (Semenza, 2001c) by the induction of genes that regulate a number of cellular processes, importantly angiogenesis and glycolysis (Semenza, 2001b). Hypoxia is a known survival signal for neutrophils (Walmsley et al., 2005).

HIF is a heterodimer consisting of two subunits: HIF-α and -β. Although both HIF-α and -β are constitutively expressed and translated, only HIF-β (also known as aryl hydrocarbon translocator or ARNT) is constitutively stable. HIF-α is an oxygen-sensitive subunit and undergoes ubiquitin-mediated proteasomal degradation under normal oxygenated conditions (Semenza, 2001a). HIF recognises and binds the core DNA sequence, 5'-RCGTG-3' (also known as hypoxia responsive elements [HREs]), within the promoter regions of a large number of genes that provide survival advantages to the cell under hypoxic conditions (Ebert and Bunn, 1998, Semenza, 1999).

Oxygen responsive regulation of HIF is shown in **Figure 1.6**. Under normal oxygen conditions (normoxia), expression of the α-subunit of HIF is modulated by prolyl hydroxylase domain-containing enzymes (PHDs), which hydroxylate at conserved proline residues residing in the degradation domain of the α-subunit (Bruick and McKnight, 2001, Epstein et al., 2001). This hydroxylated HIF-α is then readily detected by an E3-ubiquitin ligase (also called von Hippel-Lindau protein, p^{VHL}) leading to proteasomal degradation of HIF-α (Ivan et al., 2001, Jaakkola et al., 2001, Maxwell et al., 1999). HIF-α activity is also regulated by hydroxylation by factor-inhibiting HIF (FIH) (Lando et al., 2002a, Lando et al., 2002b, Mahon et al., 2001). FIH hydroxylates HIF at an asparagine residue, thus preventing the interaction of HIF with its transcriptional co-activator, p300. This results in the impairment of HIF-mediated transcriptional activity (Dames et al., 2002, Freedman et al., 2002). Under hypoxia, HIF-α is not hydroxylated, which results in p300 recruitment and prevents

proteasomal degradation since HIF-α is no longer recognised by p^{VHL}. HIFα is then translocated to the nucleus and combines with HIF-β to form a heterodimer that ultimately binds with HREs of various genes (**Figure 1.6).**

Although, some studies compare the effects of hypoxia (<4 KPa) than normoxia (19-21 KPa), which may not mirror the actual physiological or pathophysiological conditions. Neutrophils are constantly exposed to a varying degree of oxygen tensions even under normal physiology. Bone marrow, the generation site of neutrophils, is considered as a significantly hypoxic environment, where oxygen tension has been reported as low as 1.3 KPa in murine models (Spencer et al., 2014). In addition to this, mature neutrophils are rapidly and constantly exposed to a range of oxygen tensions in blood circulation: ranging from ~13 KPa in the arteries, ~7 KPa in arterioles and ~3-4 KPa in capillaries and venules. Considering the oxygen diffusion limit of capillaries, the actual oxygen tension in tissues is even lower than that of capillaries, and this is called 'physiological hypoxia' (Nolte et al., 1997, Spencer et al., 2014, Stewart et al., 1982, Zheng et al., 2015). This relative low availability of oxygen in tissue can be further amplified under conditions of inflammation and infection, as a result of increased metabolic demands and oxygen consumption by pathogens or immune cells.

It is further noteworthy to mention that whether experimental hypoxic condition mimic the condition of the lung in normal physiology or in episodes of inflammation. Since the oxygen tension of alveoli (~14 KPa) is much higher than that of other systemic organs (<3 KPa), the lung is considered an oxygen-rich organ. Thus, the oxygen pressure of a hypoxic lung (~6 KPa) is higher than that of other systemic organs (~1.3 KPa) (Frohlich et al., 2013). Therefore, the experimental condition of hypoxia at ~3 KPa may mimmic the conditions of lung epithelium, however, it would be more challenging to recapitulate the actual condition of hypoxia in the episodes of inflammation in the disease such as COPD.

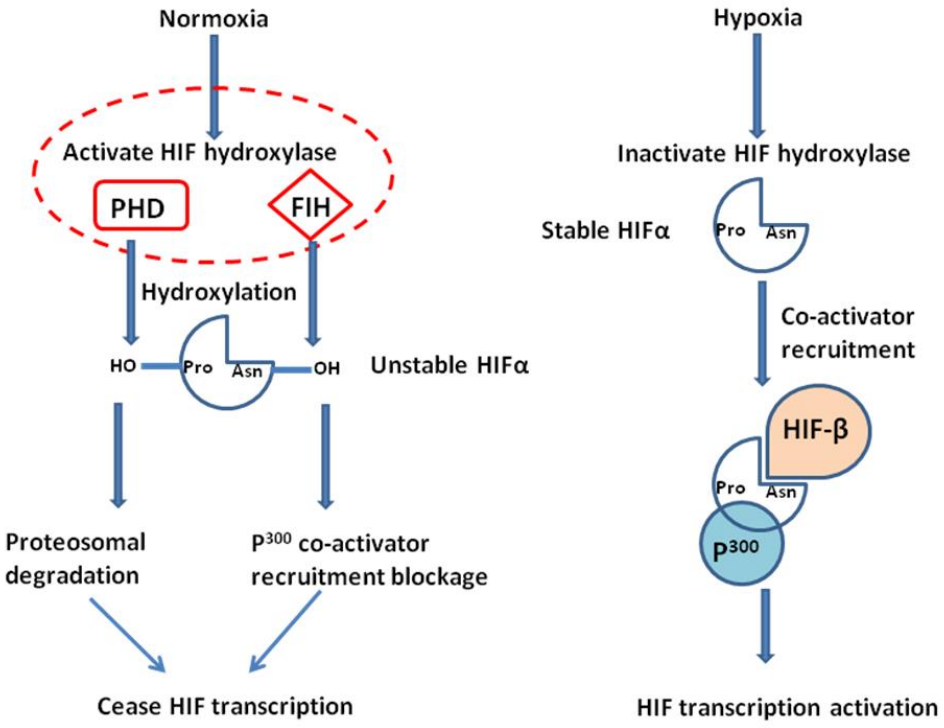


Figure 1.6. Oxygen-sensitive regulation of HIF. Under the normal oxygenated condition, HIF-α is hydroxylated at proline and asparagine residues by PHDs and FIH, respectively. Prolyl-hydroxylated HIF-α is then targeted for proteasomal degradation and the hydroxylation at an asparagine residue also prevents P^{300} co-activator recruitment, thereby switching off transcriptional activity. In a condition of hypoxia, being escaped from proteasomal degradation, HIF-α remains stable and recruits the co-activator, P^{300}; thus switching on the transcriptional activity. [This figure was recreated from (Klimova and Chandel, 2008)].

1.6. Neutrophil heterogeneity and functional plasticity in inflammation

Increasing evidence suggests that neutrophils exist as heterogenous subsets with functional plasticity (Ellett et al., 2015, Hu et al., 2014, Welin et al., 2013). The heterogeneity of neutrophils is well-characterised in the tumour microenvironment, where neutrophils have polarised phenotypes such as N1 (anti-tumorigenic and pro-inflammatory) and N2 (pro-tumorigenic and immunosuppressive) (Fridlender et al.,

2009), similar to that of macrophages M1 and M2 phenotype (Goerdt and Orfanos, 1999, Gordon, 2003, Mantovani et al., 2004). Similar polarised phenotypes are thought to exist and play important roles in infection or inflammation, although it is not well-characterised. Neutrophils subsets have been characterised by the differential expression profile of the surface receptors or the density, and are associated with multiple diseases. The expression of surface receptors is associated with a number of pathological conditions such as ICAM1 with systemic inflammation and reverse migration (Beyrau et al., 2012), CD63 in the airway with cystic fibrosis (Tirouvanziam et al., 2008) and CD177 in autoimmune disorders (Bauer et al., 2007, Hu et al., 2014). Further to neutrophil heterogeneity, neutrophil functions are also suggested to be plastic as evidenced by the ability of neutrophils to differentiate into other myeloid cells, including DCs (Scapini and Cassatella, 2014). The functional plasticity was further evidenced by a study showing the ability of bone-marrow derived neutrophils to differentiate to a hybrid cell population (which share characteristics of both neutrophils and DCs), when cultured with GMCSF (Matsushima et al., 2013). Taken together, because of the functional plasticity of neutrophils it is possible that the microenvironments (such as sites of inflammation or infection) may determine the functional fates of neutrophils.

IFN-γ/LPS stimulation is commonly used for polarisation of macrophages towards pro-inflammatory M1 phenotype (Gordon, 2003). Although IFN-γ/LPS driven neutrophil polarisation is not extensively studied, in a recent study IFN-γ/LPS was reported to have the ability to polarise neutrophils to pro-inflammatory phenotype, based on the expression profile of pro- and anti-inflammatory markers after the stimulation (Ma et al., 2016).

1.7. Therapeutic targeting of neutrophil apoptosis in inflammatory disease

Engaging neutrophil apoptosis is considered to be a potential therapeutic strategy for the resolution of inflammation in inflammatory disease. This is supported by an increasing number of studies using inhibitors of survival pathways in order to resolve inflammation *in vivo*.

1.7.1. CDK inhibitors

CDK inhibitors (such as R-Roscovitine) have been implicated as potent anti-inflammatory agents that induce neutrophil apoptosis (Rossi et al., 2006) as well as inhibiting lymphocyte proliferation and secretory function (Obligado et al., 2008). R-roscovitine augments neutrophil apoptosis by down-regulating the levels of Mcl-1in a caspase-dependent manner (Leitch et al., 2010, Rossi et al., 2006), although the exact mechanism by which R-roscovitine regulates the expression of Mcl-1 remains to be elucidated. R-roscovitine not only promotes neutrophil apoptosis but also it reverses the pro-survival effects of GMCSF, LPS and TNFα (Leitch et al., 2010, Rossi et al., 2006). The anti-inflammatory potential of CDK inhibitors in promoting neutrophil apoptosis has been suggested as therapeutically beneficial since these inhibitors resulted in improvement of clinical outcomes in murine experimental disease models of arthritis, lung fibrosis and pleurisy (Rossi et al., 2006). Recently, by using genetic and pharmacological inhibition approach in zebrafish inflammation model, CDK9 has been suggested as a potential target for controlling inflammation resolution (Hoodless et al., 2016), which was further confirmed by the ability of a CDK9 inhibitor (AT7519) to promote neutrophil apoptosis in acute respiratory distress syndrome (ARDS) (Dorward et al., 2017).

1.7.2. NF-kB and MAPK inhibitors

Several studies showed a considerable promise in targeting the NF-κB and MAPK pathways for inflammation resolution in animal models. In murine models of pleurisy,

NF-κB and ERK inhibitors were noted to promote neutrophil apoptosis and reduce the burden of other inflammatory cells (Blackwell et al., 2004, Klein et al., 2000, Sawatzky et al., 2006). Importantly, an oligonucleotide decoy to NF-κB was not only shown to promote neutrophil apoptosis but also enhance the efferocytic clearance (Maiuri et al., 2004). As NF-κB provides cues for neutrophil persistence (Ward et al., 2004), NF-κB inhibitors could be considered as therapeutic agents in resolving neutrophilic inflammation, although NF-κB has also been found to be involved in the regulation of inflammation resolution (Lawrence et al., 2001). However, independent studies show variable results, with NF-κB inhibition augmenting neutrophil apoptosis in chronic inflammation in rat models (Maiuri et al., 2004), whereas in an LPS-induced pleurisy model NF-κB inhibition failed to augment neutrophil apoptosis (Maiuri et al., 2004). To date, there is a significant and unmet need for the identification and development of therapeutics that prevent or reverse the celullar mechanisms that underpin neutrophillic inflammation.

1.8. Hypotheses and aims

I hypothesise that neutrophil function, in particular apoptosis and phagocytosis, in chronic inflammatory conditions such as COPD is dysregulated and that this contributes to persistent inflammation. I further hypothesise that novel molecular targets regulating neutrophil apoptosis are therapeutically targetable for chronic inflammatory diseases.

I will test this hypothesis by completing the following aims:

1. Study functional differences in neutrophil apoptosis and phagocytosis in patients with COPD versus healthy controls.
2. Explore inflammatory factors that regulate neutrophil function including hypoxia, ROS and other priming factors such as IFN-γ and LPS.
3. Screen a library of protein kinase inhibitor compounds in neutrophil apoptosis assays to identify protein kinases that play a role in neutrophil survival.
4. Study the role of potential kinases in neutrophil survival and inflammation *in vivo* and therefore, study kinase inhibitors for highlighting the therapeutic strategies for chronic inflammatory diseases.
5. Explore molecular mechanisms of kinase signalling in the context of neutrophil survival.

Chapter 2. Materials and methods

2.1. Study site and ethical consideration

The study was conducted in the Department of Infection, Immunity and Cardiovascular Disease, Medical School, University of Sheffield, United Kingdom. The ethical approval of taking and use of blood samples from healthy human volunteers was obtained from the South Sheffield Research Ethics Committee (reference number: STH13927). Recruitment of COPD patients was approved by the National Research Ethics Service (NRES) Committee Yorkshire and the Humber (for Sheffield cohort, reference number: 10/H1016/25) or the North West 11 Research Ethics Committee (for Manchester cohort, reference number: 10/H1016/25). Written consents from each of the study participants were obtained as a requirement of ethical guidelines.

2.2. Isolation of human neutrophils from peripheral blood

Neutrophils from peripheral blood of healthy subjects or COPD patients were isolated by dextran sedimentation and discontinuous plasma-Percoll gradients, as described previously (Haslett et al., 1985). Neutrophils are very prone to become activated, and this isolation method minimises the activation compared to other methods (Haslett et al., 1985). Appropriate care was followed throughout the procedure to avoid cell activation due to unnecessary disturbance as part of handling procedures, and also Ca^{2+}/Mg^{2+} free buffers (e.g. HBSS, Hank's Balanced Salt Solution; Gibco, Life Technologies, Paisley, UK) were used to avoid cell activation. Peripheral blood was collected and transferred to 50 ml centrifuge tubes, containing tri-sodium citrate (Martindale Pharmaceuticals, UK) as an anticoagulant. 4.4 ml of 3.8% [w/v] tri-sodium citrate was taken for each 35.6 ml of blood, to get the final concentration of 0.42%

[w/v] tri-sodium citrate. Immediately after pouring into centrifuge tubes, blood was mixed very gently with sodium citrate to prevent blood coagulation. The tubes containing the blood were then centrifuged at 270 g for 20 minutes at room temperature (MSE MISTRAL 3000i centrifuge, Sanyo, Loughborough, UK). This centrifugation resulted in the separation of a clear upper phase called platelet rich plasma (PRP) from a lower phase containing cells. The PRP was then collected carefully into fresh 50 ml centrifuge tubes and spun at 1155 g for 20 minutes at room temperature to obtain platelet poor plasma (PPP). The PPP was then collected into fresh tubes for use in a later step of the procedure. To the lower phase containing cells, 6 ml of 6% dextran (Pharmacosmos A/C, Denmark; made in 0.9% saline) was added to each tube, followed by addition of 0.9% sterile saline (Baxter Healthcare, Switzerland) up to a total volume of 50 ml. The tubes were then inverted very slowly a few times until properly mixed. Any bubbles present in the tubes and the lids were then removed using Pasteur pipette and the tubes were incubated for 25-30 minutes at room temperature to allow sedimentation of RBCs, resulting in the separation of two phases. The upper phase containing the leukocytes was then collected into fresh tubes and spun at 185 g for 6 minutes at room temperature. During this spin, the plasma-Percoll gradient was prepared. The gradient consists of two phases, the upper (42%) and the lower (51%). Plasma-Percoll phases were prepared separately by mixing 90% Percoll (v/v with saline, Sigma-Aldrich, Gillingham, UK) with PPP, obtained from an earlier stage of the preparation. For making the lower phase, 1.02 ml 90% Percoll was carefully mixed with 0.98 ml PPP and for making the upper phase, 0.84 ml 90% Percoll was mixed with 1.16 ml PPP, avoiding bubbles. By using a Pasteur pipette, 2 ml of the upper Percoll phase (42%) was then carefully overlaid onto 2 ml of the lower phase (51%) in a 15 ml centrifuge tube. The supernatant from the centrifuged upper phase was aspirated and discarded, and the soft pellet was resuspended in 2 ml of PPP. The cell suspension was then layered onto the gradient and centrifuged at 225 g for 11 minutes at room temperature, without a brake. This

allows separation of neutrophils from other leukocyte populations (as shown in **Figure 2.1**). After the centrifugation, the upper layer containing peripheral blood mononuclear cells (PBMCs) was collected first and then the middle layer containing neutrophils (and other granulocytes) was collected into tubes containing 25% (v/v) PPP in HBSS without Ca^{2+} and Mg^{2+} (Gibco, Life Technologies, Paisley, UK). Neutrophils were counted using a haemocytometer and centrifuged at room temperature for 6 minutes at 420 g. Finally, the cells were resuspended in RPMI 1640 (Gibco, Invitrogen Ltd., Paisley, UK) complete media supplemented with 10% Fetal Bovine Serum (FBS, Sigma-Aldrich, Gillingham, UK) and 1% penicillin-streptomycin (Gibco, Life Technologies, Paisley, UK) at a density of 5 x 10^6 cells/ml. The isolated neutrophils were then used in *in vitro* functional models as described. The purity of neutrophils from each preparation was calculated using differential counts by light microscopy and only neutrophil preparations with a purity ≥ 95% were used in neutrophil apoptosis (detailed in **section 2.4.2.**) and phagocytosis assays (detailed in **section 2.9. and 2.11.**).

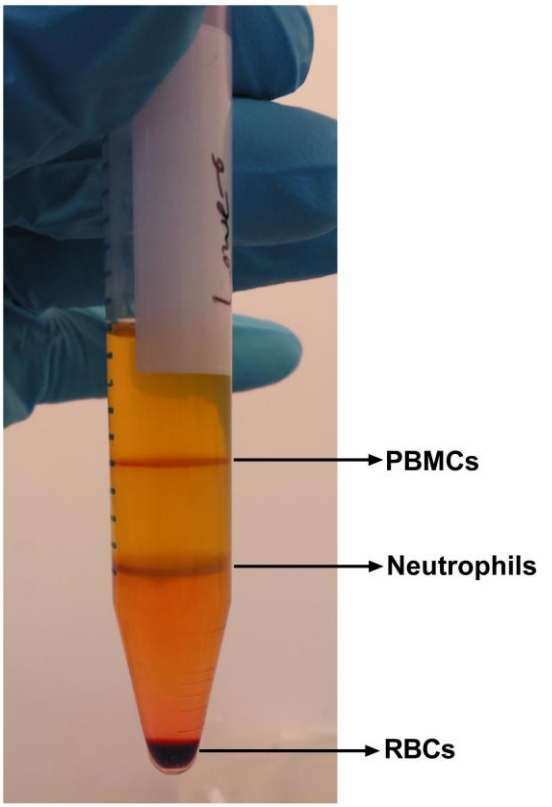


Figure 2.1. Separation of Neutrophils by percoll-gradient centrifugation. Following Percoll-gradient centrifugation, neutrophil (and other granulocyte) layer (middle) was separated from PBMC (top) and RBC (bottom).

2.3. Culturing of human neutrophils

All cells are from young healthy subjects unless otherwise specified, for the cases of COPD patients and age-matched healthy subjects. Freshly isolated neutrophils were resuspended in RPMI 1640 (Gibco, Invitrogen Ltd., Paisley, UK), supplemented with 10% FBS (Sigma-Aldrich, Gillingham, UK) and 1% penicillin-streptomycin (Sigma-Aldrich, Gillingham, UK) at a concentration of 5 x 10^6 cells/ml. Unless otherwise stated, 0.25 x 10^6 neutrophils were plated into each well of 96-wells flexible, untreated polyvinyl chloride general assay plates (Corning, Sigma-Aldrich, Gillingham, UK) in the presence or absence of the following reagents: protein kinase inhibitor

compounds from PKIS (Published Kinase Inhibitor Set, GlaxoSmithKline), GMCSF (PeproTech, Inc), LPS (*E. coli* LPS, Sigma-Aldrich), N^6- Monobutyryladenosine- 3', 5'- cyclic monophosphate (N^6-MB-cAMP, Biolog), Erbstatin analog (Cayman Chemicals), Tyrphostin AG825 (Sigma-Aldrich), Q-VD-OPh hydrate (R&D System), Pyocyanin (prepared as previously described (Usher et al., 2002)), IFN-γ (PeproTech), LPS (*E. coli* LPS, Sigma-Aldrich), mito-TEMPO (Sigma-Aldrich), Sulforaphane (Sigma-Aldrich). Plates were incubated at 37°C/5%CO_2 in a humidified CO_2 incubator (Sanyo Electric Co Ltd., Japan).

2.3.1. Neutrophil culture in hypoxia

For hypoxia studies, normoxic [P_{O2}≈19 KPa] cells were incubated as described above. Hypoxic cells were incubated in Invivo2 400 hypoxic chamber [P_{O2}≈3 KPa] (Ruskinn, Bridgend, UK). Cells plated in hypoxic conditions were resuspended in RPMI media supplemented with 10% FBS and 1% penicillin-streptomycin, which was pre-equilibrated before use for at least 3 hours in the hypoxic chamber to ensure the media to be hypoxic. A blood gas analyser (ABLS, Radiometer, Copenhagen, Denmark) was used to monitor the level of pH and the partial pressure of CO_2 and O_2 to ensure the consistency of these parameters within the hypoxic chamber.

2.4. Assessment of neutrophil Apoptosis

The rates of neutrophil apoptosis upon culturing neutrophils were assessed by flow cytometry based on binding of apoptotic neutrophils with Annexin-V/Topro-3, and/or light microscopy based on morphological changes of apoptotic neutrophils.

2.4.1. Flow cytometric examination of neutrophil apoptosis

To assess apoptosis, neutrophils were stained with PE-conjugated annexin-V (BD Pharmingen, BD Biosciences, Oxford, UK) and Topro-3 (Molecular Probes, Life Technologies, Paisley, UK) to identify apoptotic neutrophils by flow cytometry.

Annexin-V specifically binds with membrane phospholipid, phosphatidyl serine (PS) that irreversibly flips out of the plasma membrane of apoptotic cells, whereas Topro-3 binds with DNA of late apoptotic/necrotic cells. Following *in vitro* culture, neutrophils were resuspended and transferred to 1.5 ml microcentrifuge tubes followed by centrifugation at 425 g for 2 minutes at 4^{o}C using table-top centrifuge (Centrifuge 5417R, Helena Biosciences, Sunderland, UK). Cells were then washed in PBS at the same centrifugation speed. After removing the supernatants, cells were resuspended in 47.5 µl of 1x Annexin binding buffer (ABB, BD Pharmingen, BD Biosciences, Oxford, UK). PE-conjugated annexin-V was then added at a dilution of 1 to 20 (2.5 µl added to 47.5 µl cell suspension in each tube) and the tubes were incubated for 20 minutes on ice in the dark. Negative controls, unstained and EDTA negative controls were used in each of the experiments as follows. The binding of annexin-V to PS is calcium dependent and thereby, the addition of EDTA [0.02 M, Sigma-Aldrich, Gillingham, UK] inhibits annexin-V binding to apoptotic cells. Single stain controls for both annexin-V and Topro-3 were also included in the assay. During the period of incubation with annexin-V, Topro-3 (1:7500 v/v) was prepared in ABB, and 150 µl of the diluted Topro-3 was added to the dual stained and Topro-3 only tubes. To each of the unstained or annexin-V only tubes, 150 µl of ABB was added. Sample acquisition was performed using the Attune flow cytometer (Attune, Applied Biosystems, Life Technologies, Paisley, UK). Neutrophils were gated from forward (FSC) and side (SSC) scatter properties, and 10,000 events from the neutrophil gate were recorded for each of the samples. Flow data were analysed using flowJo (FlowJo, LLC). The percentage of annexin-V and Topro-3 positive cells was assessed from a quadrant plot drawn from the gated neutrophil population, and the apoptosis was calculated by the summation of annexin-V positive and annexin-V/Topro-3 double positive events.

In kinase inhibitor compound library screening experiments, neutrophil apoptosis was measured as above but in a 96 wells plate format instead of 1.5 ml centrifuge tubes,

which allowed for a high throughput system. Briefly, after the incubation period with inhibitor compounds was complete, the plate was centrifuged at 420 g for 6 minutes to pellet the cells and was washed once with PBS. Cells were then stained with PE-conjugated annexin-V and Topro-3, as described above. Flow cytometry acquisition of cells was performed by Attune Flow cytometer using a built-in Auto sampler, which acquires samples from 96 wells plate in a robotic manner based on pre-set program. The percent apoptosis was calculated as described above. The total cell count was also estimated from respective gates.

2.4.2. Morphological examination of neutrophil apoptosis

In addition to the flow cytometry based detection, neutrophil apoptosis was also assessed by light microscopy and distinguished morphologically from healthy neutrophils (Savill and Haslett, 1995, Savill et al., 1989a). The main characteristic morphologic feature of apoptotic neutrophils is a condensed nucleus, as opposed to the classic multi-lobed nuclei seen in healthy neutrophils (Savill and Haslett, 1995).

2.4.2.1. Making cytocentrifuge slides for light microscopy

Microscopic slides were labelled appropriately and placed onto cytospin holders (Shandon Life Sciences International Ltd., Runcorn, UK). Then 'filter cards for Shandon cytospin' (Thermo Scientific, UK) were placed onto the slides in such a way that the smooth side of the filters faced the slides. Cytospin chambers (Shandon Life Sciences International Ltd., Runcorn, UK) were placed on top of the filter paper and the holders were clipped to secure the components. After incubation period was complete, neutrophils in each well were resuspended and the entire volume (approx. 100 µl or the equivalent of 250,000 cells) applied to the cytospin chambers in an upright position. Cells were then spun at 300 rpm for 3 minutes (for freshly isolated neutrophils, 400 rpm for 3 minutes) in a cytocentrifuge machine (Shandon Life Sciences International Ltd., Runcorn, UK). After performing the cytocentrifugation, the slides were disassembled and allowed to dry for 1-2 minutes. Then the slides were

fixed with methanol and allowed to dry completely. Finally, the slides were stained first with Quik-diff red (Reagena Ltd., Toivala, Finland) for about 1 minute, and then with Quik-diff blue (Reagena Ltd., Toivala, Finland) until they were visibly stained (2-5 minutes). The slides were then rinsed under tap water to remove excess dye followed by leaving them on the bench to dry. This staining procedure was based on an H & E (Hematoxylin & Eosin) staining principle. The hematoxylin binds with acidic cellular structure notably nucleic acid within the nucleus leading to purple staining whereas the eosin binds with basic cellular components (mainly cytoplasmic proteins) that results in pink staining within the cytoplasm (Fischer et al., 2008). Once dried after the staining, a drop of DPX mounting medium (Sigma-Aldrich, Gillingham, UK) was added onto each slide in a fume hood, followed by fixing the slides with cover slips. The slides were then left overnight in a fume hood to be dried completely and the slides were then ready to view under an inverted light microscope (Nikon Eclipse TE300, Japan) at a magnification of 100X. The apoptotic and healthy cells were counted based on morphologically distinguishable characteristics as mentioned above (**Figure 2.2**). A total of 300 cells were counted for each slide for light microscopy for differential counts and assessment of apoptosis and phagocytosis.

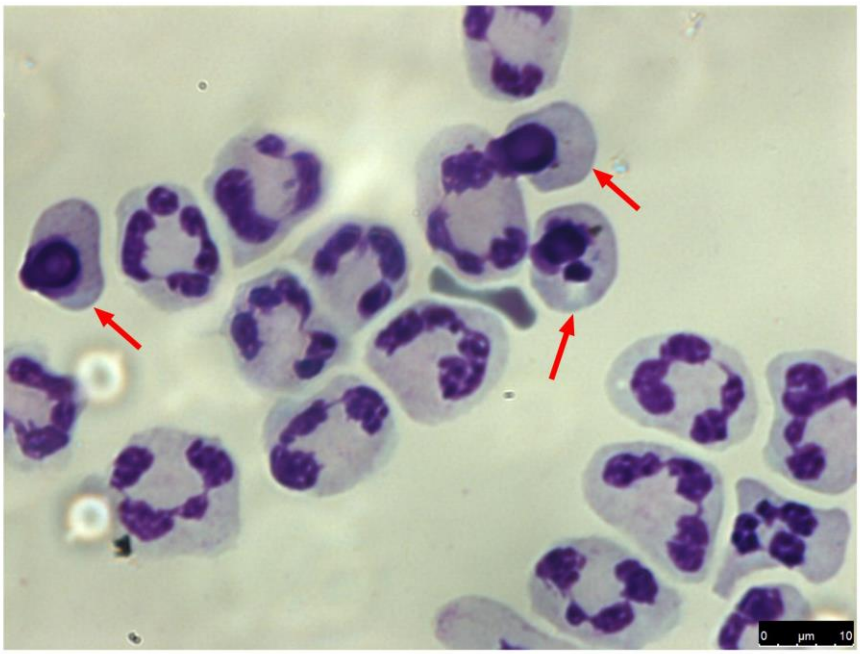


Figure 2.2. Apoptotic neutrophils. A photomicrograph representing apoptotic and healthy neutrophils after culturing for 6 hours. After the incubation period, neutrophils were cytocentrifuged, fixed with methanol and stained with Quick-Diff red and blue stain (H & E). The red arrows highlight apoptotic neutrophils and the remaining neutrophils are viable.

2.5. Detection of mitochondrial ROS (mROS)

To determine the effect of sulforaphane (Sigma-Aldrich) and mito-TEMPO (Sigma-Aldrich) on mitochondrial ROS (mROS) production, neutrophils were stained with mitosox (Molecular probes, Life Technologies, Paisley, UK). Mitosox is a red dye which can be targeted specifically to mitochondria and acts as a probe for mROS production. Annexin V-APC (BD Pharmingen, BD Biosciences, Oxford, UK) was used to gate the viable neutrophils. Neutrophils were cultured with or without sulforaphane (100 nM) and mito-TEMPO (100 µM) in a 37^{o}C incubator for 4 hours. Just 30 minutes before the end of incubation period, mitosox (5 µM) was added. The plates were then wrapped with foil to avoid light exposure and incubated for 30 minutes in the cell

culture incubator. After the incubation, cells were then transferred to microcentrifuge tubes and centrifuged at 425 g for 2 minutes at 4^{o}C. After discarding the supernatants, the cells were resuspended in annexin binding buffer. Annexin V-APC (1:20 dilution) was then added and mixed properly. Cells were then incubated for 20 minutes at dark on ice. When the incubation is complete, cells were transferred to respective FACS tubes to run through LSRII flow cytometer (BD Biosciences, California, USA). Live neutrophils were gated from FSC and SSC and 10,000 events from neutrophils population were recorded for each of the samples. Data were analysed using flow data analysis software, FlowJo (FlowJo, LLC). Mean fluorescence intensity (MFI) was calculated by comparing the value of geometric mean intensity with that of mitosox negative control (only annexin V-APC).

2.6. Western blotting

2.6.1. Preparation of whole cell lysates

Neutrophils (5 x 10^6 cells in each condition) were cultured with appropriate stimuli according to the experimental settings. After the incubation period, cells were resuspended and transferred to 1.5 ml microcentrifuge tubes. Cells were centrifuged at 425 g for 2 minutes and pellets were washed once with 1X PBS. Whole cell lysates were prepared by resuspending neutrophils (5 x 10^6 neutrophils) in 50 µl ice-cold hypotonic lysis buffer (**Appendix 7.1**) and incubating the tubes for 1 minute on ice for complete lysis to occur. Then 50 µl 2X SDS (Sodium dodecyl sulfate, Sigma-Aldrich) lysis buffer (**Appendix 7.2**) was added to each tube followed by heating the mixture at 95^{o}C for 10 minutes on a heat block. The whole cell lysates were then stored at -20^{o}C until use.

2.6.2. SDS-PAGE (SDS polyacrylamide gel electrophoresis)

SDS-PAGE is a well-established protein separation technique which separates protein based on molecular size. Briefly, gel casting apparatus were wiped with 70% ethonol and assembled according to the manufacturer instructions. Both resolving (12% acrylamide) and stacking (4% acrylamide) gels were prepared in separate 50 ml tubes, as per the recipe in **Table 2.1**. TEMED initiates polymerisation of acrylamide and so was added immediately before pouring the gel in the cell casting apparatus. The resolving gel was poured in the cast and isopropanol was overlaid to prevent the gel from drying out. Once solidified (approx. 15-20 minutes), the isopropanol was removed and rinsed with tap water. The stacking gel mixture was then pipetted onto the resolving gel and combs to create samples wells were inserted. Once solidified, the gels were then placed into a running tank with 1X running buffer (**Appendix 7.3**) and combs were carefully removed. Protein samples were loaded into each well including a protein ladder (molecular weight marker, Geneflow Ltd, UK). The electrical current of 80 V was then applied until the samples migrated beyond the stacking gel following which the current was increased up to 160 V until the visible bromophenol blue passed through the entire gel.

Table 2.1. Recipe for 12% SDS-PAGE resolving and 4% stacking gels.

Reagents	12% Resolving gel (4 gels)	4% stacking gel (4 gels)
Water	16.5 ml	12 ml
40% Acrylamide (Geneflow Ltd., UK)	11.3 ml	2480 µl
1.5 M Tris PH 8.0 (Sigma-Aldrich, UK)	9.5 ml	-
0.5 M Tris PH 6.8 (Sigma-Aldrich, UK)	-	5040 µl
20% SDS (Fisher Scientific, UK)	187.5 µl	100 µl
20% APS (Sigma-Aldrich, UK)	375 µl	200 µl
TEMED (Sigma-Aldrich, UK)	15 µl	20 µl

2.6.3. Blotting (Semi-dry electrotransfer)

Separated proteins from the SDS-PAGE gel were electrotransfered to a PVDF (polyvinylidene difluoride)-membrane (Bio-rad Laboratories Ltd., UK) by semi-dry transfer method using Trans-Blot®-Turbo™ transfer system (Bio-rad Laboratories Ltd). Briefly, after electrophoresis, the gels were disassembled from the glass plates and placed in transfer buffer (**Appendix 7.3**). PVDF membranes were activated by soaking for 1-2 minutes in methanol and placed into transfer buffer. Then transfer 'sandwich' was constructed whereby the gel and PVDF membranes are sandwiched by blotting papers (Whatman International, Maidstone, UK) as follows. PVDF membranes were placed onto 3 pieces blotting papers that have already been pre-soaked in transfer buffer and the gels were then carefully positioned onto the PVDF membrane followed by putting 3 pieces of pre-soaked blotting papers onto the gel. Any air bubbles trapped within the stack were then removed by rolling a stripette over the top, and finally transfer of proteins was performed by Trans-Blot®-Turbo™ transfer system (program set at constant 25 V 1.5 amp for 25 minutes).

2.6.4. Immunostaining and detection of proteins

After transfer was complete, PVDF membranes were blocked in 5% skimmed milk power (Sigma-Aldrich) in 1X TBS-tween (**Appendix 7.3**) on a rotating platform for one hour at room temperature, followed by washing the membranes once in TBS-tween for 5 minutes. Membranes were then probed with primary antibodies to p-AKT (1:800, Cell Signalling Technology), AKT (1:1000, Cell signalling Technology), Mcl-1 (1:300, Santa Cruz Biotechnology) or P38 (1:2000, StressMarq Biosciences Inc.). Primary antibodies were made up in 5% skimmed milk in TBS-tween and co-incubated with membranes overnight at 4ºC on a rolling platform. After the incubation period, the membranes were washed with 1X TBS-tween 3 times for 10 minutes each followed by incubating with horseradish peroxidase-conjugated secondary antibodies

(Dako, made in 5% skimmed milk/1X TBS-tween) for one hour at room temperature. Finally, immunoreactive products were detected with chemiluminescent substrate solution ECL2 (GE Healthcare) and visualised by ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad Laboratories Ltd.). All immunoreactive bands in the blots showing in the figure are at predicted molecular weight of proteins of interest. All the primary and secondary antibodies and their dilution are listed in **Appendix 7.4**.

2.7. Zebrafish *in vivo* model

2.7.1. Fish husbandry

Neutrophil specific GFP transgenic zebrafish line, Tg (mpx:GFP)i114, (simply referred to as mpx: GFP) was raised and maintained according to standard protocols (Renshaw et al., 2006) in UK Home Office approved aquaria in the Bateson Centre at the University of Sheffield. Adult fish are maintained in the storage tank (approx. 40 fishes each tank) in 14 hours light and 10 hours dark cycle at 28^{o}C. Fish larvae were obtained by pairing (marbling) between male and female fish. Briefly, one male and one female adult fishes were collected from the storage tank, and placed into a marbled covered container and kept them together to mate to get zebrafish eggs (Alternatively, containers with ridged bottom can also be used for paring between male and female fishes and eggs are collected from between ridges). The following day, eggs were collected into a fresh petri dish containing conditional medium, E3 (**Appendix 7.5**) and fish larvae were born the same day. Since larvae are transparent, neutrophils are visible as GFP-positive cells under a fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.7.2. Zebrafish tail injury model of inflammation

The mpx:GFP zebrafish line was used to elicit inflammatory response by tail transection as described previously (Elks et al., 2011, Renshaw et al., 2006), and

then the number of neutrophils at site of injury was determined at 2, 4, 6, 8 and 24 hours post injury (hpi) by counting GFP-positive neutrophils. Briefly, zebrafish larvae were maintained in the E3 medium. 3 days post-fertilised larvae were anesthetized in 0.168 mg/ml Tricaine (3-amino benzoic acid ethyl ester, Sigma-Aldrich) in a petri dish and after ensuring cessation of their complete movement, their tails were transected with a microscalpel at an area distal to the circulatory loop within the tail (Elks et al., 2011, Renshaw et al., 2006) (site of tail transection shown in **Figure 5.21**). Following injury, as part of an inflammatory response, neutrophils migrate to the injured tissue, and therefore the number of neutrophils at the site of injury was counted at 2, 4, 6, 8 and 24 hours post injury. During each time point, the larvae were anaesthetized with tricaine and the numbers of neutrophils were counted (blinded to the experimental conditions) under an inverted fluorescent microscope. Following counting after each time point, the tricaine containing medium was replaced with fresh E3 medium and larvae moved back into the incubator.

For Tyrphostin AG825 experiment, following treating 2 days post fertilized larvae with Tyrphostin AG825 [10 µM, Sigma-Aldrich] for 24 hours in a 6-well plate, followed by tail transection as above. The tricaine containing media was then replaced with fresh E3 medium with Tyrphostin AG825 at the same concentration and the plate was incubated at 28^{o}C. The neutrophil numbers were counted at 4 and 8 hpi.

2.7.3. Whole body neutrophil count

To enumerate neutrophils across the whole body, uninjured larvae were treated with Tyrphostin AG825 for 24 hours and then mounted in 0.8% low-melting-point agarose (Sigma-Aldrich) followed by imaging. GFP-positive neutrophils were subsequently counted across the whole body in high-resolution images as follows. Briefly, 2 days post fertilized larvae were treated with Tyrphostin AG825 (or DMSO) in 6 well culture plates. Thirty minutes before completing the incubation, 0.8% low-melting agarose (Sigma-Aldrich) with tricaine (4.2%) was then made in E3 medium and kept in a water

bath (pre-heated at 42^{o}C) in order to keep the agarose in a liquid state and tolerable to fish larvae. Larvae were anesthetised with tricaine and transferred into an imaging chamber (up to 8 larvae per chamber). After removing media from the chamber, low-melting agarose was then poured into the chamber and larvae were rapidly lined up, so that they can be imaged from a lateral view before the agarose gel solidifies. Once solidified, the immobilised larvae were then imaged by a fluorescent microscope (Eclipse TE2000-U, Nikon) at 4X magnification for the detection of GFP-positive neutrophils. Numbers of neutrophils were then counted, blind with respect to experimental conditions, from the captured images.

2.8. Lung injury murine model

Approval of working with murine model [ISP reference: AART13, PPL number: 40/3726, PPL holder: David Dockrell] was obtained from the Animal Welfare and Ethical Review Body at the University of Sheffield, UK. All handling of, and procedures on live mice was kindly performed by Dr. A. A. Roger Thompson and Ms. Lynne Williams. LPS-induced lung injury murine model has been used extensively to study neutrophilic inflammatory response in the lung (Faffe et al., 2000, Lefort et al., 2001, Menezes et al., 2005, Thompson et al., 2013).

2.8.1. LPS nebulisation and Tyrphostin AG825 injection

C57BL/6 mice [female, 9-10 weeks old] were nebulised with LPS from *Pseudomonas aeruginosa* (Sigma-Aldrich) to induce neutrophilic inflammation in the lung. Briefly, 16 mice were moved from their cages and split between two plastic containers where nebulisation was performed by connecting the container to an oxygen driven nebuliser. Oxygen was delivered to the containers (at 6 litres/minute) through an LPS solution (600 µl of 5 mg/ml LPS to deliver a total of 3 mg per group of mice), which converted the LPS into aerosol for inhalation. After all of the LPS was nebulised, the

mice were moved back to their respective cages. Soon after the nebulisation process, the mice were injected intraperitoneally with Tyrphostin AG825 (Tocris Bioscience) at 20 mg/kg in 10% DMSO-vegetable oil [Tyr group, 8 mice] or equal volume of 10% DMSO-vegetable oil [control group, 8 mice], and then transferred to their original cages for 48 hours.

2.8.2. Bronchoalveolar lavage (BAL) collection

After 48 hours of Tyrphostin AG825 (or DMSO) treatment, mice were sacrificed by terminal anaesthesia (pentobarbitone administered by intraperitoneal route) and then BAL sampling was performed on 8 mice per group (Tyrphostin AG825 and DMSO). Briefly, after exposing the chest wall by opening the skin from neck to the abdomen, pneumothoraxes were introduced via wounding the inferior surface of the diaphragm. Heart and lung were then exposed by cutting along the lateral margin of rib cages on each side. The trachea was then uncovered, connected to a cannula, and 3.5 ml ice cold 0.9% saline (0.5-1 ml in each aliquots) was then introduced and the lavage was collected in 15 ml centrifuge tubes. The lavage was maintained on ice while the exact volume was measured. The total number of cells in each lavage was determined by a haemocytometer. The samples were then centrifuged at 1000 g for 5 minutes followed by removing the supernatants. Cell pellets were then resuspended in FBS at 2×10^6 cells/ml. The equivalent of 0.6×10^6 cells were cytocentrifuged (2 cytospins) and stained with Quick-Diff (see **section 2.4.2.1.**) for differential cell counts, morphological assessment of neutrophil apoptosis and macrophage efferocytosis. For validation of neutrophil apoptosis by flow cytometry, the equivalent of 0.2×10^6 cells were also stained with PE-conjugated Annexin-V and Topro-3 (see detail in **section 2.4.1.**).

2.9. Zymosan A phagocytosis

On the day of the experiment, 10 mg Zymosan A (Sigma-Aldrich, UK) particles was mixed with 1 ml of non-heat inactivated FBS in a microcentrifuge tube and agitated for 1 hour at 37^{o}C for opsonisation. After the incubation, the particles were then washed 3 times with sterile PBS followed by resuspension in PBS at a concentration of 30 mg/ml, and this stock was then further diluted 1 to 5 times to get the working stock (6 mg/ml). In suspension, the zymosan A particles form aggregates of about 3 µm in diameter. Neutrophils were pre-incubated in the presence or absence of a number of stimuli (mito-TEMPO or IFN-γ/LPS or hypoxia) as discussed in chapter 3. After the incubation, zymosan A particles (0.2 mg/ml) were added to each of the wells to be tested [5 µl from 6 mg/ml working stock was added to each well in a total volume 150 µl] and plates were incubated for 15 minutes at 37^{o}C to undergo phagocytosis. The phagocytosis process was stopped by quickly placing the plates on ice. The samples were then cytocentrifuged onto microscopic slides at 300 rpm for 3 minutes as described above in **section 2.4.2.1.** The slides were then fixed, stained, and prepared for morphological identification. Under the light microscope, 300 cells were counted for assessing the ingestion of zymosan A particles by neutrophils. Phagocytic Index (PI) was then calculated by multiplying the mean number of zymosan A particles per neutrophil with the percent of neutrophils containing the particles.

2.10*. E. coli* phagocytosis assay

To assess the effect of IFN-γ/LPS stimuli on neutrophil phagocytosis, flow cytometry based detection of phagocytosis was adopted by using *E. coli*-Alexafluor®488 (Molecular Probes, Life Technologies, Paisley, UK) and *E. coli*-pHrodo green bioparticles conjugate (Molecular Probes, Life Technologies, Paisley, UK). Briefly, neutrophils were primed with IFN-γ (100 ng/ml) for 1 hour followed by addition of *E.*

coli LPS (100 ng/ml) for an additional 3 hours. A plate for 4ºC control was also used in parallel, which allow subtracting any background signals coming from the adherence of uningested bacteria on the surface of neutrophils. Once the incubation period was complete, 4ºC control plate was placed into the fridge, before addition of *E. coli*-Alexafluor®488 or *E. coli*-pHrodo green bioparticles.

2.10.1. Detection by E. coli-Alexafluor®488bioparticles

Once the incubation period was complete, heat-killed *E. coli*-Alexafluor®488 bioparticles was added to each of the wells at MOI=1, except the wells with no *E. coli* and the plates were then incubated for 45 minutes at 37ºC. The incubation of 4ºC control plate was performed at 4ºC in fridge. After the incubation, the plates were placed on ice to stop further phagocytosis. The cells were suspended and transferred to labelled and ice-chilled microcentrifuge tubes followed by a centrifugation at 425 g for 2 minutes at 4ºC. After discarding the supernatants, the cells were then resuspended in PBS followed by another centrifugation at the same rate as mentioned above. After second centrifugation, the supernatants were then discarded and the cells were finally resuspended in 300 µl 2% FCS-PBS to perform sample acquisition using Attune flow cytometer (Attune, Applied Biosystems, Life Technologies, Paisley, UK). As a set parameter, 10,000 events were recorded from neutrophil gate for each of the samples. Alexafluor®488 is excited by 488 laser and its emission spectra is detected by BL1-A detector. Data were analysed using the analysis software, flowJo (FlowJo, LLC). Neutrophils were gated from the forward and the side scatter plot and the geometric means of BL1-positive signals from neutrophil population were determined by plotting histogram with BL1-signals. To get actual signals for phagocytosis, geometric mean for 4ºC control was subtracted from geometric mean under each condition.

2.10.2. Detection by E. coli-pHrodo green bioparticles

After 4 hour incubation, *E. coli*–pHrodo green bioparticles (0.01 mg) were added to each of the wells to be tested, except the no *E. coli* control wells. The pHrodo is a better indicator of phagocytosis as it provides viable signals only under acidic pH and therefore the bacteria present only within acidified phagolysosomes are detected. To get maximum acidification neutrophils were then incubated for 1.5 hours at 37^{o}C, whereas 4^{o}C control plate was incubated at fridge at 4^{o}C. After the incubation, the cells were then washed in PBS as mentioned above. Finally, the cells were resuspended in 300 µl PBS and run through Attune flow cytometer. Like Alexafluor®488, the pHrodo green was also excited by 488 laser and its emission was detected by the same BL1-A detector. Data analyses were performed using FlowJo as mentioned above. Geometric means of BL1-A positive signals were used as a measure of phagocytosis.

2.11. Phagocytosis assay with heat-killed *Staphylococcus aureus*

S. aureus phagocytosis assay was performed by adding heat-killed *S. aureus* (SH1000 strain at MOI=5 & 10) to neutrophils for 30 minutes. After the incubation period was complete, the assay plates were placed on ice to stop further phagocytosis. Cytocentrifuge slides were then prepared as described in **section 3.4.2.1.** Phagocytic index was calculated by morphologically counting the number of neutrophils with or without the bacteria and also the total number of bacteria present within neutrophils. 300 cells were counted for each condition.

2.12. Data analysis and statistics

Data were analysed using GraphPad Prism 7 (GraphPadSoftware, San Diego, CA) using one way ANOVA (with appropriate post-test adjustment) for all multivariable

comparisons. For comparison between two conditions, analysing cells from the same subject, paired t-test was performed. Non-parametric t-test (Mann-Whitney U-test) was performed for comparison between two groups in *in vivo* experiments. Flow cytometry data were analysed by flow data analysis software, FlowJo. Data are expressed as mean ± SEM (Standard Error of Mean), and $P<0.5$ are accepted to be significant, with a 95% confidence interval.

Chapter 3: Modulation of neutrophil phagocytosis by inflammatory mediators

3.1. Brief Introduction

The clearance of bacteria and other inhaled particles is an important physiological process in the lung to maintain sterility. The sterility is maintained by various mechanisms including mucociliary clearance, secreted anti-microbial proteins and the presence of immune cells in the lung (Hiemstra, 2006, Holmskov et al., 2003, Sallenave, 2002). Although resident airway macrophages are important phagocytes, neutrophils are rapidly recruited to the airway and play roles in phagocytic defence in this context (Zhang et al., 2000). There is emerging evidence that innate immunity is compromised in airway diseases including COPD, leading to bacterial persistence and colonization of the airways, ultimately contributing to disease pathophysiology (Berenson et al., 2006, McClure and Schiller, 1996). The phagocytic capacity of macrophages has been extensively studied in COPD, and a large number of studies suggest that macrophages in these patients are less efficient at bacterial phagocytosis than healthy macrophages (Donnelly and Barnes, 2012). Macrophage efferocytosis, which shares similarities with phagocytosis, was also noted to be depressed in COPD (Henson et al., 2006, Hodge et al., 2003). Less is known about neutrophil phagocytosis defects in COPD and since bacterial persistence is common in this patient group, it is possible that a phagocytosis defect is also present in neutrophils. Identification of defects in neutrophil phagocytosis and elucidating the mechanisms of the defects could provide potential therapeutic opportunities for treating COPD and other airway diseases. There are very few reports studying the difference in phagocytosis between COPD and healthy neutrophils and the reported results are conflicting; some studies show reduced phagocytosis (Fietta et al., 1988, Prieto et al., 2001) whereas others show unaltered phagocytosis (Muns et al., 1995, Walton, 2014). Here, I studied the phagocytosis capacity of neutrophils from COPD

patients versus healthy controls. Since ROS-driven oxidative stress (Kirkham and Barnes, 2013) and IFN-γ production (Hens et al., 2008, Panzner et al., 2003, Reeves et al., 2010) are both increased in COPD, and are considered as key contributors to COPD pathophysiology, these pro-inflammatory mediators may regulate neutrophil functions, notably phagocytosis. Furthermore, since tissue hypoxia is a characteristic feature of COPD (Han and Mallampalli, 2015, Hoenderdos and Condliffe, 2013) and is known to regulate neutrophils apoptosis (Elks et al., 2011, Walmsley et al., 2011) and respiratory burst activity (McGovern et al., 2011), hypoxia may also regulate neutrophil phagocytosis. Therefore, the effects of these pro-inflammatory factors (IFN-γ, ROS and hypoxia) on neutrophil phagocytosis were studied *in vitro.*

3.2. Results

3.2.1. Circulatory neutrophils from COPD patients had a modest phagocytic defect that was not corrected by Nrf2 agonist Sulforaphane.

Here, I compared the phagocytic potential of neutrophils from COPD patients and healthy subjects. Demographic characteristics of COPD patients (obtained from the Manchester Cohort, COPD MAP) are presented in **table 3.1.** Phagocytosis was assessed by adding heat-killed *Staphylococcus aureus* (SH1000 strain, MOI=5 &10) to freshly isolated neutrophils from COPD patients and healthy control subjects for 30 minutes followed by assessment of phagocytosis by light microscopy (**Figure 3.1 A**). Of note, the doses of *S. aureus* and 30 minutes cut off time for phagocytosis were chosen based on a personal communication and an existing study on *S. aureus* phagocytosis by neutrophils (Lu et al., 2014). The phagocytic response was assessed in terms of phagocytic index, % of neutrophils containing *S. aureus* and the mean number of *S. aureus* per neutrophil (**Figure 3.1 B-D**). The phagocytic index was calculated by multiplying the percent of neutrophils containing the bacteria with the mean bacteria per neutrophil. The phagocytic index was found to be significantly

higher in healthy neutrophils compared to COPD neutrophils at MOI 10 (p=0.03) (**Figure 3.1 B**). However, the actual measured parameters, the percent neutrophils containing the bacteria and the mean bacteria per neutrophil, were unchanged (p=0.29 and p=0.41, respectively) (**Figure 3.1 C-D**).

Table 3.1. Demographic characteristics of COPD patients (Machester Cohort). Data are expressed as mean ± SEM or number.

Number of patients (n)	10
Age years (yrs)	66.4 ± 1.9
Female/Male (n)	4/6
FEV1 (L)	1.7 ± 0.2
FEV1 (%)	63.9 ± 7.0
FEV1/FVC (%)	52.5 ± 4.6
Smoking Status (Current/Ex)	2/8
Pack years	50.1 ± 6.2
Exacerbation in last 1 year	1.4 ± 0.5

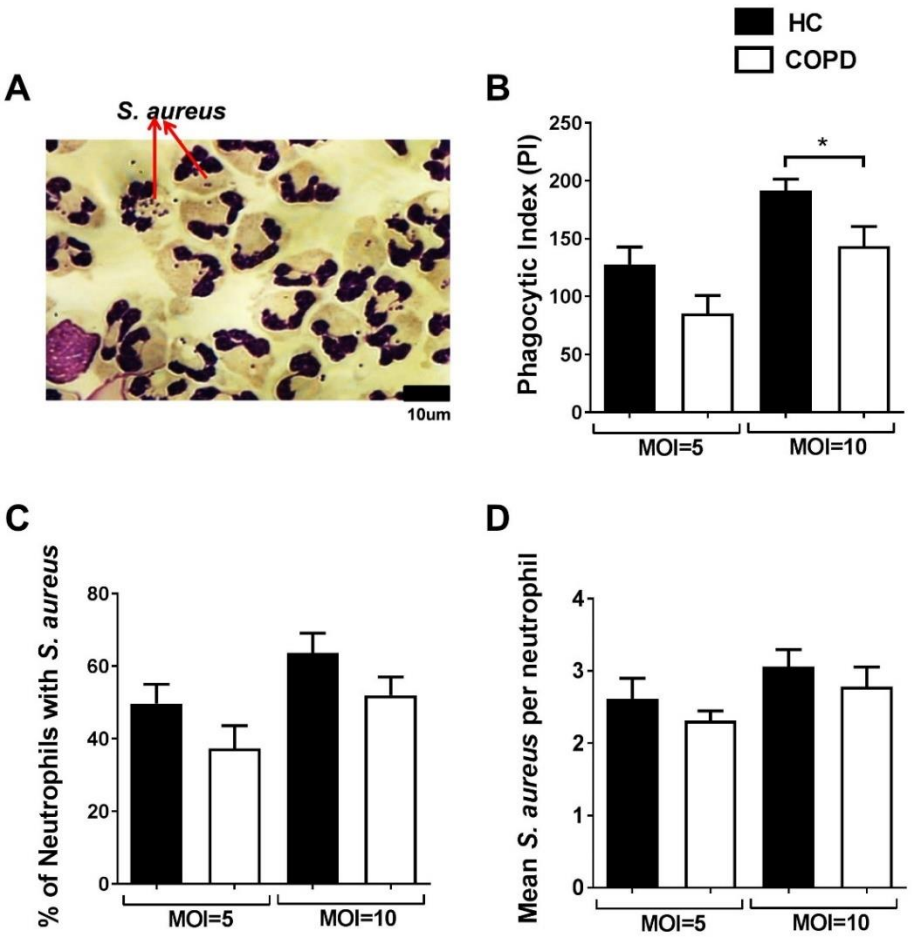


Figure 3.1. Circulatory neutrophils from COPD patients have a modest phagocytic defect. Neutrophils were isolated from COPD patients (COPD) and healthy subjects (HC) followed by an assessment of phagocytosis by adding heat-killed *S. aureus* (SH1000 strain, MOI 5 & 10) for 30 minutes. (A) A representative slide picture showing the internalised bacteria within neutrophils under an inverted light microscope. (B) A graph showing the phagocytic index which was calculated by multiplying the percent of neutrophils containing *S. aureus* with the mean *S. aureus* per neutrophil. (C & D) Graphs representing the % neutrophils containing *S. aureus* and the mean number of *S. aureus* per neutrophil, respectively, are displayed. Data are expressed as mean ± SEM (standard error of mean) with duplicate measurements under each condition, n=5 (COPD), 4 (HC). The closed and open bars represent HC and COPD, respectively. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by a non-parametric t-test (Mann-Whitney U test), and indicated as *$p<0.05$.

Oxidative stress is a key factor in the pathogenicity of COPD (Kirkham and Barnes, 2013), is linked with macrophage phagocytic defects in this disease (Harvey et al., 2011, Hodge et al., 2011, Vecchiarelli et al., 1994), and the use of anti-oxidants has been suggested to improve this defect (Harvey et al., 2011, Hodge et al., 2011, Vecchiarelli et al., 1994). Here, I hypothesised that upregulation of cellular anti-oxidants may also have potential in improving the neutrophil phagocytosis defect. To test this hypothesis, neutrophils from COPD patients and healthy volunteers were incubated with or without the Nrf2 agonist sulforaphane [SLF, 0.1, 1.0, 10 μM] for 4 hours (a time point where SLF reduces mROS, detailed below in **section 3.2.3**) followed by phagocytosis of heat-killed *S. aureus* for 30 minutes as above. Of note, Nrf2 is a transcription factor that stimulates upregulation of over 200 anti-oxidant and detoxifying genes such as glutathione reductase (GSR), glucose-6-phosphate dehydrogenase (G6PD), heme oxygenase-1 (HO-1) and NADPH: quinone oxidoreductase 1 (NQO1) (Gorrini et al., 2013, Kobayashi and Yamamoto, 2006). Phagocytosis was found to be reduced in neutrophils from COPD patients compared to healthy subjects (phagocytic indices for COPD and HC were 90.74±38.29 and 209.90±61.23, respectively), although not statistically significant because of variability between experiments ($p=0.33$), and this was not changed by sulforaphane treatment ($p>0.99$) (**Figure 3.2**).

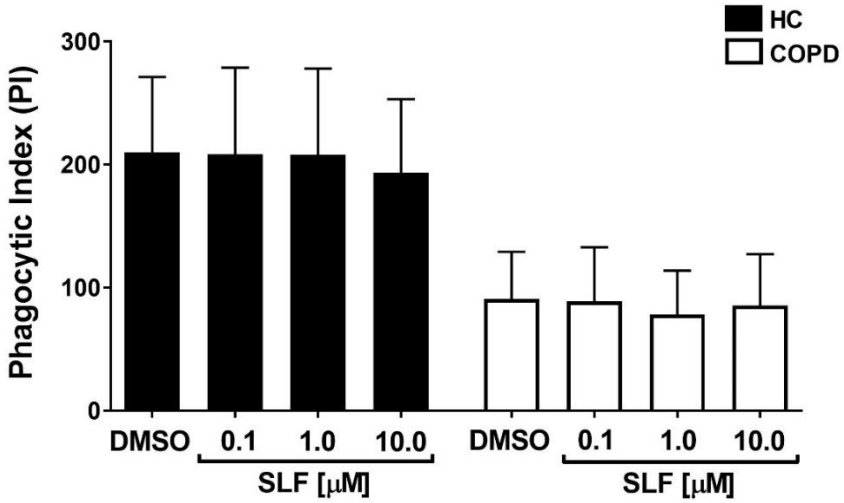


Figure 3.2. Nrf2 activator Sulforaphane did not correct the neutrophil phagocytic defect. Neutrophils from COPD patients (COPD) and healthy subjects (HC) were pre-treated with or without sulforaphane [SLF, 0, 0.1, 1.0, 10 μM] for 4 hours followed by an assessment of phagocytosis by adding heat-killed *S. aureus* (SH1000 strain, MOI 10) for 30 minutes. The phagocytic ability was expressed as the phagocytic index and assessed by counting the neutrophils (with or without the bacteria) under a light microscope. The phagocytic index was calculated by multiplying the percent of neutrophils containing *S. aureus* with the mean *S. aureus* per neutrophil. Data are expressed as mean ± SEM with duplicate measurements under each condition, n=5 (COPD), 4 (HC). The closed and open bars represent HC and COPD, respectively. DMSO is the vehicle control for sulforaphane. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significance was calculated by one way ANOVA (with Bonferroni's post-hoc test).

3.2.2. Scavenging mitochondrial ROS (mROS) with mito-TEMPO did not change neutrophil phagocytic capacity:

The generation of mROS has been implicated in cellular signalling (Finkel, 2012) and associated with multiple disease pathologies (Li et al., 2013, Naik and Dixit, 2011), including COPD (Wiegman et al., 2015). Furthermore, mROS generated from the activation of TLR 2, 3 & 4 via the translocation of mitochondria to the macrophage phagosome has been reported to promote bacterial killing (West et al., 2011).

Importantly, mROS has also been noted to regulate macrophage phagocytosis (Dehn et al., 2016). Neutrophils have relatively few functional mitochondria (Maianski et al., 2004) and little is known about the role of mROS in neutrophil phagocytosis. To investigate a potential role of mROS in neutrophil phagocytosis, the neutrophils were incubated in the presence or absence of mROS scavenger, mito-TEMPO [100 µM] followed by adopting two phagocytosis assays: zymosan A and *S. aureus* phagocytosis assays. Of note, the 100 µM concentration of mito-TEMPO was chosen based on existing studies with macrophages and lung cancer cells (Heid et al., 2013, Pan et al., 2013).

Zymosan is a polysaccharide extract from yeast cell walls, and has been used in experimental phagocytosis models for many decades (Di Carlo and Fiore, 1958). Following the incubation of neutrophils with or without mito-TEMPO [100 µM] for an hour, zymosan A phagocytosis was measured by adding zymosan A particles [0.2 mg/ml] to neutrophils for 15 minutes. Of note, the dose of zymosan A particles and 15 minutes cut off time for phagocytosis was decided based on a personal communication with group members and an existing study with neutrophils (Thompson et al., 2013). Phagocytosis of zymosan A particles was measured by light microscopy and evaluated in terms of phagocytic index, % of neutrophils containing zymosan A particles and mean number of zymosan A particles per neutrophil. Uptake of zymosan A particles is shown in **Figure 3.3 A.** Mito-TEMPO pretreatment induced a small but significant reduction in the percent of neutrophils containing zymosan A ($p=0.04$) (**Figure 3.3 C**). Mito-TEMPO did not alter the phagocytosis evaluated by phagocytic index or mean zymosan particles ($p=0.16$ and $p=0.22$, respectively) **(Figure 3.3 A-D)**

In order to further validate a role for mROS, neutrophils were pre-treated with mito-TEMPO [100 µM] for a longer pre-treatment period (four hours, a treatment condition where our group have shown the ability of mito-TEMPO in the reduction of mROS;

detailed in **section 3.2.3.**) followed by addition of heat-killed *S. aureus* (at MOI 5 & 10). Phagocytic activity was assessed as above. The phagocytic index, the percent of neutrophils containing *S. aureus* and the mean *S. aureus* per neutrophil were unaltered in the presence of mito-TEMPO ($p=0.39$ and $p>0.99$; $p>0.99$ and $p>0.99$; $p=0.36$ and $p>0.99$, respectively for MOI 5 and 10) **(Figure 3.4 A-C)**.

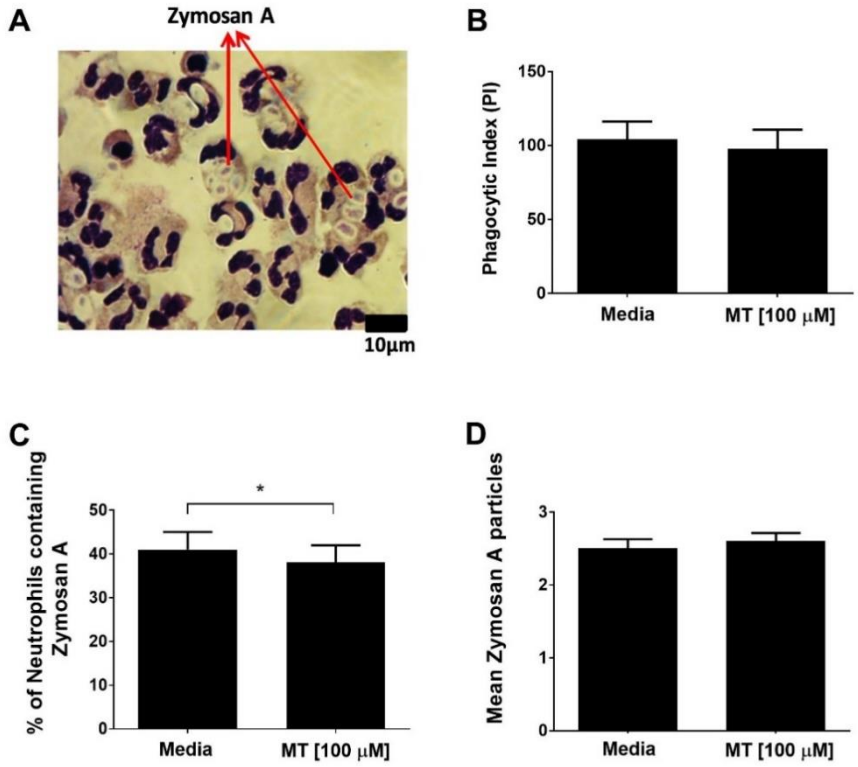


Figure 3.3. Scavenging mROS did not affect Zymosan A phagocytosis. Neutrophils from healthy subjects were pre-treated with or without a mROS scacenger mito-TEMPO [100 µM] for an hour followed by an assessment of phagocytic activity by the addition of zymosan A particles [0.2 mg/ml] to neutrophils for 15 minutes. (A) A slide picture representing the uptake of zymosan A particles (as indicated by the red arrows) by neutrophils observed under light microscope is shown. Neutrophil phagocytosis of zymosan A particles was evaluated in terms of phagocytic index (B), % of neutrophils containing the zymosan A particles (C) and the mean zymosan A particles per neutrophil (D); as presented in the graphs. Phagocytic index was calculated by multiplying the percent of neutrophils containing zymosan A with the mean zymosan A particles per neutrophil. Data represents twelve independent experiments with triplicate measurements under each condition, and the values are expressed as mean ± SEM. Media is the negative control for the assay. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by paired t-test, and indicated as *$p<0.05$.

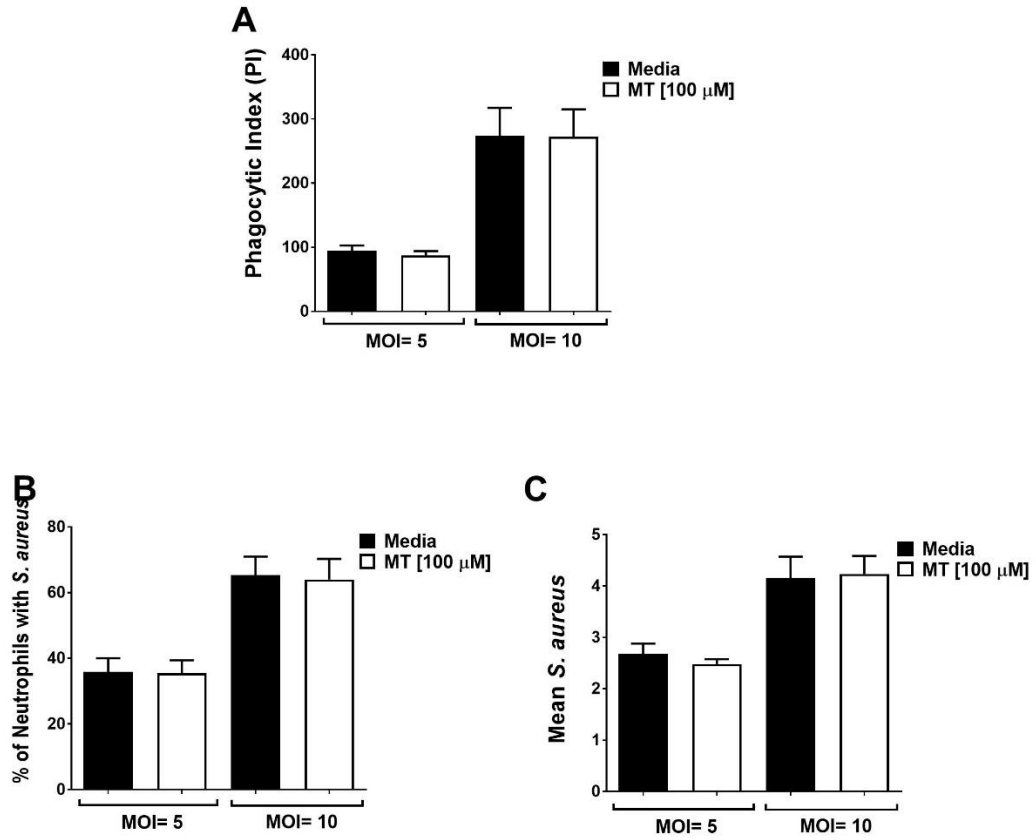


Figure 3.4. Scavenging mROS did not affect *Staphylococcus aureus* phagocytosis. Neutrophils were incubated with or without mito-TEMPO [MT, 100 μM] for 4 hours followed by an assessment of phagocytosis by adding heat-killed *S. aureus* (SH1000 strain, MOI 5 & 10) for 30 minutes. The Phagocytosis ability was calculated by counting the neutrophils (with or without the bacteria) by light microscopy. (A) A graph showing the phagocytic index, which was calculated by multiplying the percent of neutrophils containing the bacteria with the mean bacteria per neutrophil. (B&C) Graphs showing the percent of neutrophils containing *S. aureus* and the mean number of *S. aureus* per neutrophil are presented, respectively. Data represents three independent experiments with duplicate measurements under each condition and the values are expressed as mean ± SEM. The closed and open bars represent media (negative control) and Mito-TEMPO, respectively. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significance was calculated by one way ANOVA (Bonferroni's post-hoc test).

3.2.3. Sulforaphane and mito-TEMPO reduce mROS production.

To assess the effectiveness of sulforaphane [100 nM] and mito-TEMPO [100 µM] in reducing cellular mROS, neutrophils were incubated with or without these inhibitors for 4 hours followed by staining with mitosox (a mitochondrial ROS probe) and measuring the levels of mROS by flow cytometry. The mean fluorescence intensity (MFI) as a measure of mROS production was assessed. Both sulforaphane and mito-TEMPO reduced MFI values, visualised on representative histograms as a shift to the left **(Figure 3.5 A-B).** MFI values (representing overall mROS production) confirmed that sulforaphane and mito-TEMPO significantly decreased mROS production ($p=0.02$ and $p=0.03$, respectively) **(Figure 3.5 C-D)**. These data were kindly generated by an undergraduate project student named Adam Smith.

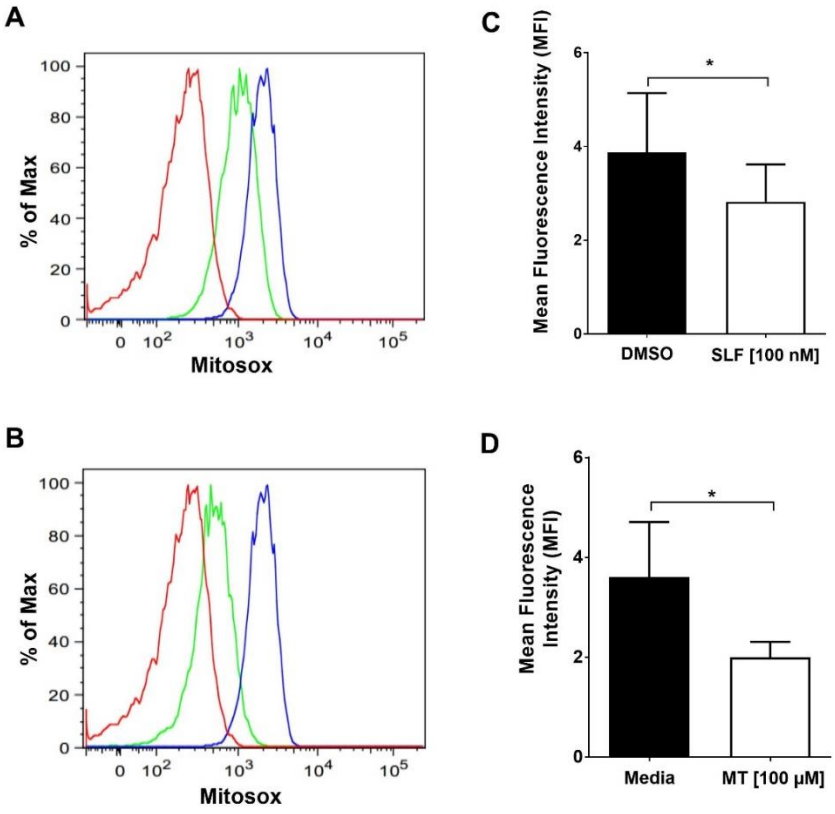


Figure 3.5. Both sulforaphane and mito-TEMPO reduces mROS. Neutrophils were incubated in the presence or absence of sulforaphane [100 nM] or mito-TEMPO [100 µM] for four hours, stained with mitosox (a probe for mROS) followed by a detection of mROS by flow cytometry. (A) Representative histograms showing the effect of sulforaphane on mROS (red, blue and green colours represent unstained, stained untreated and stained sulforaphane treated, respectively). (B) Representative histograms showing the effect of mito-TEMPO on mROS (red, blue and green colours represent unstained, stained untreated and stained mito-TEMPO treated, respectively). Both sulforaphane [100 nM] (C) and mito-TEMPO [100 µM] (D) pre-treatment significantly reduced mROS. The Mean Fluorescence Intensity (MFI) are expressed as a ratio of the intensities of mitosox stained and unstained control. Data represent 6 (C) or 7 (D) experiments with triplicate measurements under each condition, and the values were expressed as mean ± SEM. DMSO is the vehicle control for sulforaphane; media is the negative control for mito-TEMPO. Statistical significances were calculated by ratio paired-t test, and indicated as *$p<0.05$. The data were kindly generated by an undergraduate project student named Adam Smith.

3.2.4. IFN-γ and LPS-stimuli did not change neutrophil phagocytic capacity. Since IFN-γ signalling is elevated in COPD patients, particularly during acute exacerbations (Grumelli et al., 2004, Hens et al., 2008, Makris et al., 2008, Panzner et al., 2003, Reeves et al., 2010, Singh et al., 2010) and also LPS is a common pro-inflammatory factor in the lung, studying these two pro-inflammatory stimuli on immune cell functions is of great importance. Macrophages are heterogeneous and have two polarised phenotypes: M1 (pro-inflammatory) and M2 (anti-inflammatory) and IFN-γ/LPS have an ability to shift the macrophages towards an M1 phenotype (Goerdt and Orfanos, 1999, Gordon, 2003, Mantovani et al., 2004). In contrast to the expected M1 polarisation, alveolar macrophages in COPD patients has demonstrated to exhibit M2 characteristics, which has been implicated in the pathogenicity of COPD (Shaykhiev et al., 2009). Depending on the environmental cues, IFN-γ treatment has been demonstrated to induce diverse cellular and functional responses in neutrophils (Ellis and Beaman, 2004). Previously it has been shown that similar to macrophages IFN-γ/LPS has the ability to polarise neutrophils towards a pro-inflammatory N1 phenotype *in vitro* (Ma et al., 2016). Therefore, I hypothesise that IFN-γ/LPS treated neutrophils may have different or altered functional capacities, such as alteration in phagocytic capacities. To do this, neutrophils were primed with IFN-γ [100 ng/ml] alone for one hour followed by the addition of LPS [100 ng/ml] for a further 3 hours, followed by assessment of phagocytosis of zymosan A, *E. coli* and *S. aureus*. Of note, the above-mentioned doses and time points for IFN-γ/LPS treatment in these experiments were chosen since our group has shown that following the same treatment neutrophils upregulate HIF-1α, which was reported as a key regulator of macrophage phagocytosis (Anand et al., 2007).

Phagocytosis was evaluated in terms of phagocytic index, % of neutrophils with zymosan A particles and the mean zymosan A particles as described above **(Figure 3.6).** Compared to untreated control, IFN-γ/LPS did not change phagocytic index

(p=0.19) **(Figure 3.6 A).** Neither the percent of zymosan A particles nor the mean zymosan A particle per neutrophil was modified in response to IFN-γ/LPS treatment (p=0.19 and p=0.33, respectively) **(Figure 3.6 B-C).**

The effect of IFN-γ/LPS on phagocytosis of the Gram-negative bacterial pathogen *E. coli* was also studied. The phagocytosis was assessed by flow cytometry using two fluorescently labelled *E. coli* conjugates: Alexafluor®488 conjugated *E.coli* and pHrodo green conjugated *E. coli* **(Figure 3.7 &3.8).** Of note, pHrodo is a dye that produces fluorescence only under acidic conditions, for example within phagosome, which makes it as an ideal tool for studying bacterial internalisation. In the Alexafluor®488-*E.coli* phagocytosis assay a well-demarcated Alexafluor®488 signal was observed as shown in the histogram and the pseudo-colour dot plot **(Figure 3.7A&C,** respectively)**.** A 4^{o}C control was used to identify signals coming from the adherence of bacteria on the surface of neutrophils and this value was subtracted from each of the experimental conditions (IFN-γ/LPS treated and untreated). Neither the Alexafluor®488 generated GMI (Geometric Mean Intensity) nor the percent of neutrophils with *E. coli*–Alexafluor®488 was affected by IFN-γ/LPS treatment (p=0.52 and p=0.98, respectively) **(Figure 3.7 B&D).** In the same manner, a well-demarcated separation of pHrodo green fluorescence from the unstained control was observed in the pHrodo green-*E. coli* phagocytosis assay **(Figure 3.8 A&C).** Compared to the Alexafluor®488, pHrodo green dye had very little fluorescence signal in the 4^{o}C assay control as pHrodo dye should only generate a fluorescent signal in the acidified phagosome. Neither the GMI nor the percent of neutrophils containing *E. coli* –pHrodo green was affected by IFN-γ/LPS treatment **(Figure 3.8 B&D).**

To follow on from this, phagocytosis of a clinically relevant Gram-positive pathogen, *S. aureus, was also* assessed by adding heat-killed bacteria (SH1000 strain, MOI=5 & 10) for 30 minutes followed by enumerating the phagocytic index, % neutrophils containing *S. aureus* and the mean bacteria per neutrophils as above. The phagocytic

index, the percent of neutrophils containing *S. aureus* and the mean *S. aureus* per neutrophil were unaltered in the presence of IFN-γ/LPS (p=0.56 and p>0.99; p>0.99 and p>0.99; p=0.32 and p>0.99, respectively for MOI 5 and 10) **(Figure 3.9 A-C)**.

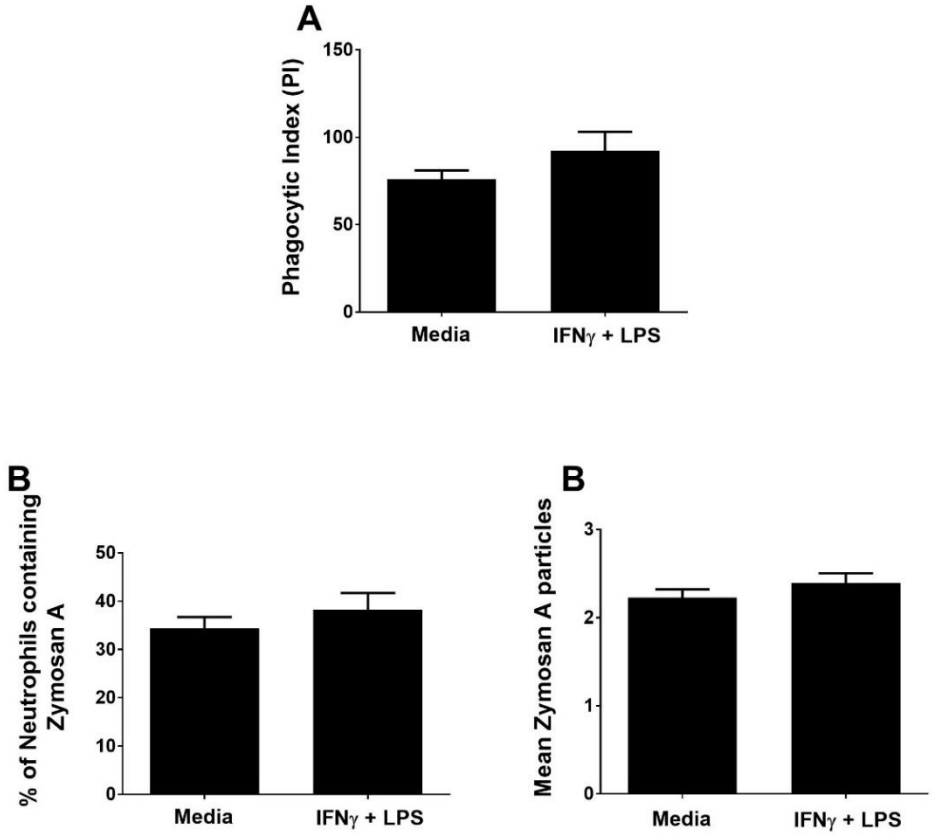


Figure 3.6. IFN-γ/LPS did not affect zymosan A phagocytosis. Neutrophils were primed with IFN-γ [100 ng/ml] for one hour followed by the addition of LPS [100 ng/ml] for a further 3 hours. Neutrophil phagocytosis was then assessed by addition zymosan A (0.2 mg/ml) particles for 15 minutes. Neutrophil phagocytosis of zymosan A particles was evaluated in terms of phagocytic index (A), % of neutrophils containing zymosan A particles (B) and Mean zymosan A particles per neutrophil (C). Phagocytic index was calculated by multiplying the percent of neutrophils containing zymosan A with the mean zymosan A particles per neutrophil. Data represent eleven experiments with triplicate measurements under each condition, and the values are expressed as mean ± SEM. Media is the negative control for IFN-γ/LPS. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significance was calculated by paired-t test.

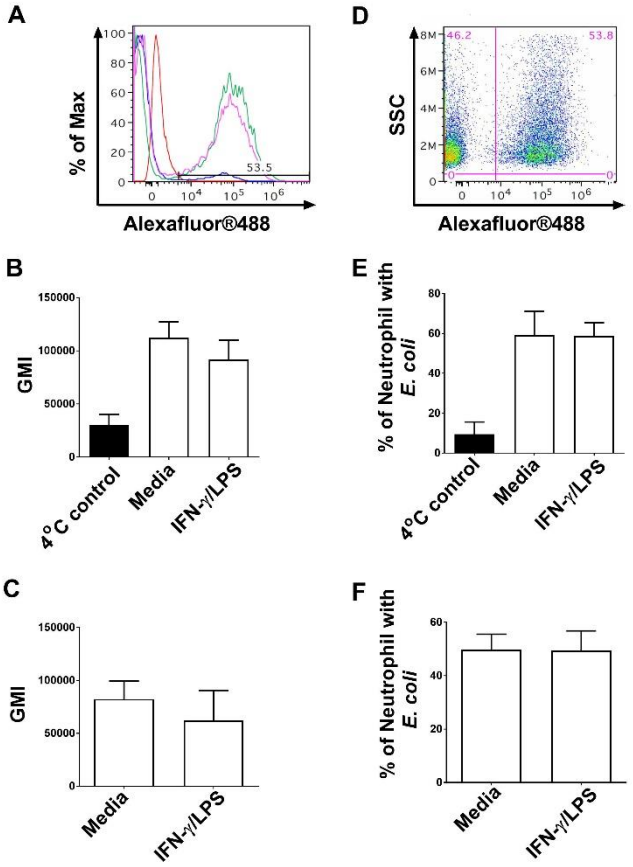


Figure 3.7. IFN-γ/LPS did not affect phagocytosis of *E. coli*-Alexafluor®488 bioparticles. Neutrophils were primed with IFN-γ [100 ng/ml] for one hour followed by the addition of LPS [100 ng/ml] for a further 3 hours. The phagocytosis was then assessed by incubating the neutrophils with *E. coli*-Alexafluor®488 for 45 minutes followed by a flow cytometric measurement of fluorescence intensity as detected by the BL1-A. (A) A representative histogram showing a well demarcated separation of Alexafluor®488 signal [unstained=red, 4ºC control=blue, untreated=green, IFN-γ/LPS treated=pink)] (B) A graph showing the geometric mean of intensity (GMI) generated from *E. coli*-Alexafluor®488 positive neutrophils. (C) A graph representing GMI after subtracting the 4ºC control signal. (D) A representative pseudo-colour plot depicting the percent of *E. coli*-Alexafluor®488 positive neutrophils. (E) A graph showing the percent of neutrophils with *E. Coli*-Alexafluor®488. (F) The percent of neutrophils with *E. Coli*-Alexafluor®488 after subtracting the 4ºC control signal. Media is the negative control for IFN-γ/LPS. Data represent experiments with triplicate measurements under each condition, and the values were presented as mean ± SEM. Statistical significance was calculated by paired-t test.

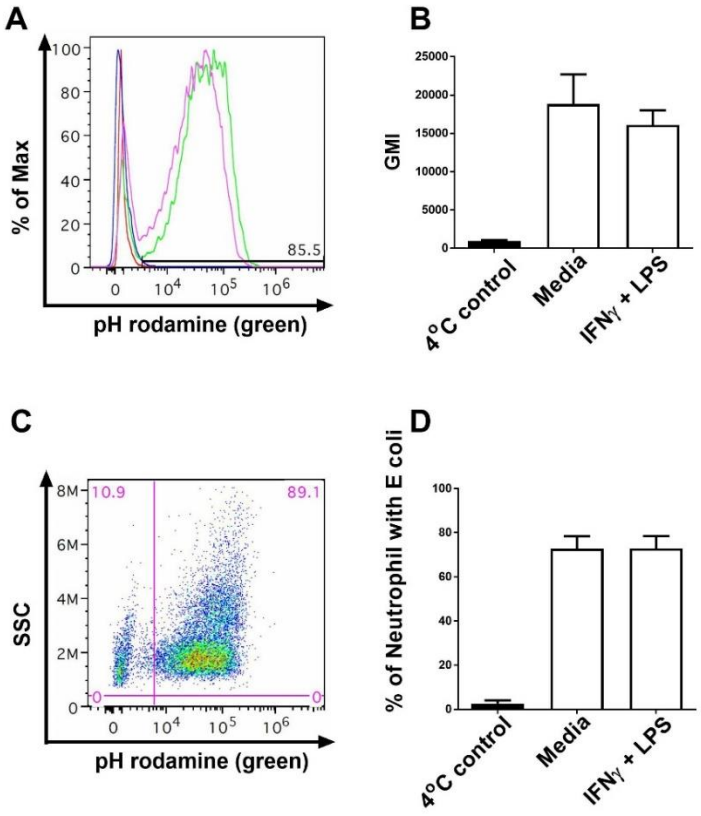


Figure 3.8. IFN-γ/LPS did not affect phagocytosis of *E. coli*-pHrodo bioparticles. Neutrophils were primed with IFN-γ [100 ng/ml] for one hour followed by the addition of LPS [100 ng/ml] for a further 3 hours. The phagocytosis was then assessed by incubating the neutrophils with *E. coli*-pHrodo green bioparticles for 90 minutes followed by a flow cytometric measurement of pHrodo fluorescence intensity detected by the BL1-A detector. (A) A representative histogram showing a well demarcated separation of pHrodo signal [unstained=red, 4°C control=blue, untreated=green, IFN-γ/LPS treated=pink)]. (B) A graph showing the geometric mean of intensity (GMI) generated from *E. coli* labelled pHrodo positive neutrophils. (C) A representative pseudo-colour plot depicting the percent of *E. coli*- pHrodo positive neutrophils. (D) A graph showing the percent of neutrophils with *E. Coli*-pHrodo bioparticles. Data represent five independent experiments with triplicate measurements under each condition, and the values are presented as mean ± SEM. Media is the negative control for IFN-γ/LPS. Statistical significance was calculated by paired-t test.

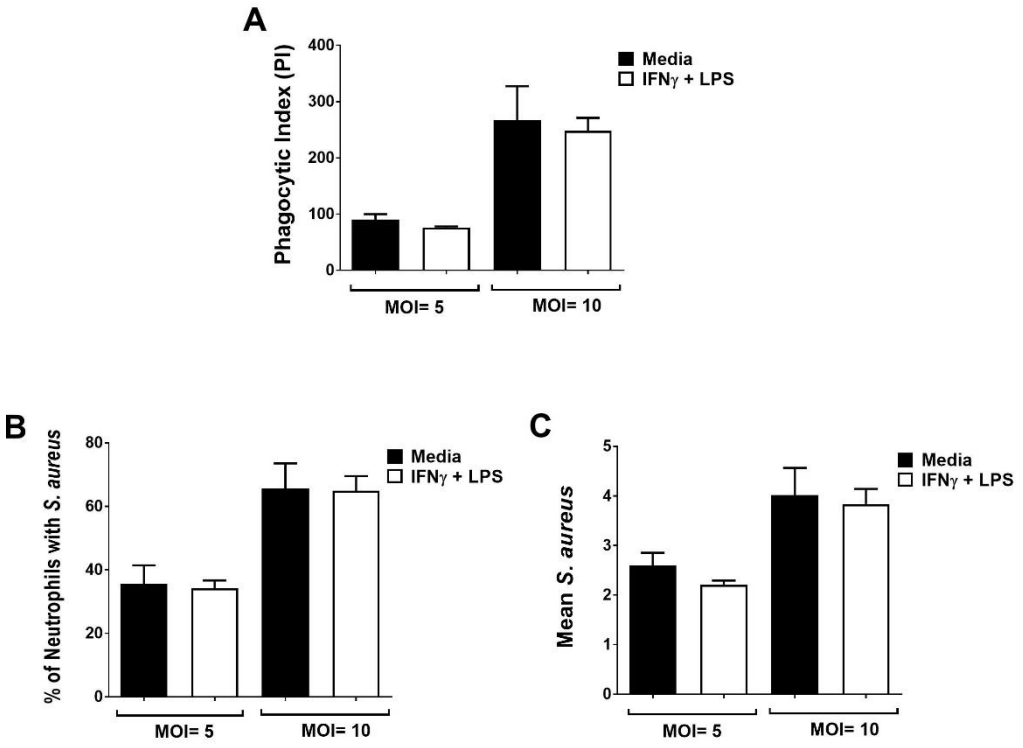


Figure 3.9. IFN-γ priming did not affect *S. aureus* phagocytosis. Neutrophils were primed with IFN-γ [100 ng/ml] for one hour followed by the addition of LPS [100 ng/ml] for a further 3 hours. The phagocytosis was then assessed by incubating the neutrophils with heat-killed *S. aureus* (SH1000 strain, MOI 5 &10). The phagocytic index (A), the percent of neutrophils containing *S. aureus* (B) and the mean bacteria per neutrophil (C) are shown in the graphical presentation. The phagocytic index was calculated by multiplying the percent of neutrophils containing *S. aureus* with the mean *S. aureus* per neutrophil. The data represent three independent experiments with duplicate measurements under each condition, and the values are presented as mean ± SEM. The closed and open bars represent media (negative control) and IFN-γ/LPS, respectively. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significance was calculated by one way ANOVA (with Bonferroni's post-hoc test).

3.2.5: Effects of hypoxia on neutrophil phagocytosis.

Tissue hypoxia is a frequent characteristic feature in inflammatory states (Taylor and McElwain, 2010), including acute and chronic lung diseases, such as COPD (Han and Mallampalli, 2015, Hoenderdos and Condliffe, 2013). It has been reported that hypoxia is a key survival stimulus for neutrophils (Walmsley et al., 2005) and thought to be a key contributor in neutrophil dominant inflammation, for example in lung inflammation in COPD. Here, I hypothesise that hypoxia may regulate neutrophil phagocytosis. To address the issue, neutrophils were incubated in hypoxia (Po_2≈3 KPa) and normoxia (Po_2≈19KPa) for 4 hours followed by an assessment of zymosan A or *S. aureus* phagocytosis. As described above, the phagocytosis of zymosan A particles or *S. aureus* was measured by light microscopy, and evaluated in terms of phagocytic index, % of neutrophils containing prey particles and the mean number of prey particles per neutrophil**.** Interestingly, hypoxia was found to have a small but significantly enhance the percent phagocytosis of zymosan A particles (p=0.04, % neutrophils containing the bacteria changed from 34.38±3.26 to 40.54±3.26), however, neither the phagocytic index nor the mean particles per neutrophil were changed (p=0.14 and p=0.33, respectively) **(Figure 3.10 A-C).** The Phagocytosis of *S. aureus* evaluated by any means was found to be comparable in normoxia and hypoxia (p=0.24 and p>0.99; p=0.18 and p=0.79; p>0.99 and p>0.99, for MOI 5 and 10, respectively) **(Figure 3.10 D-F)**.

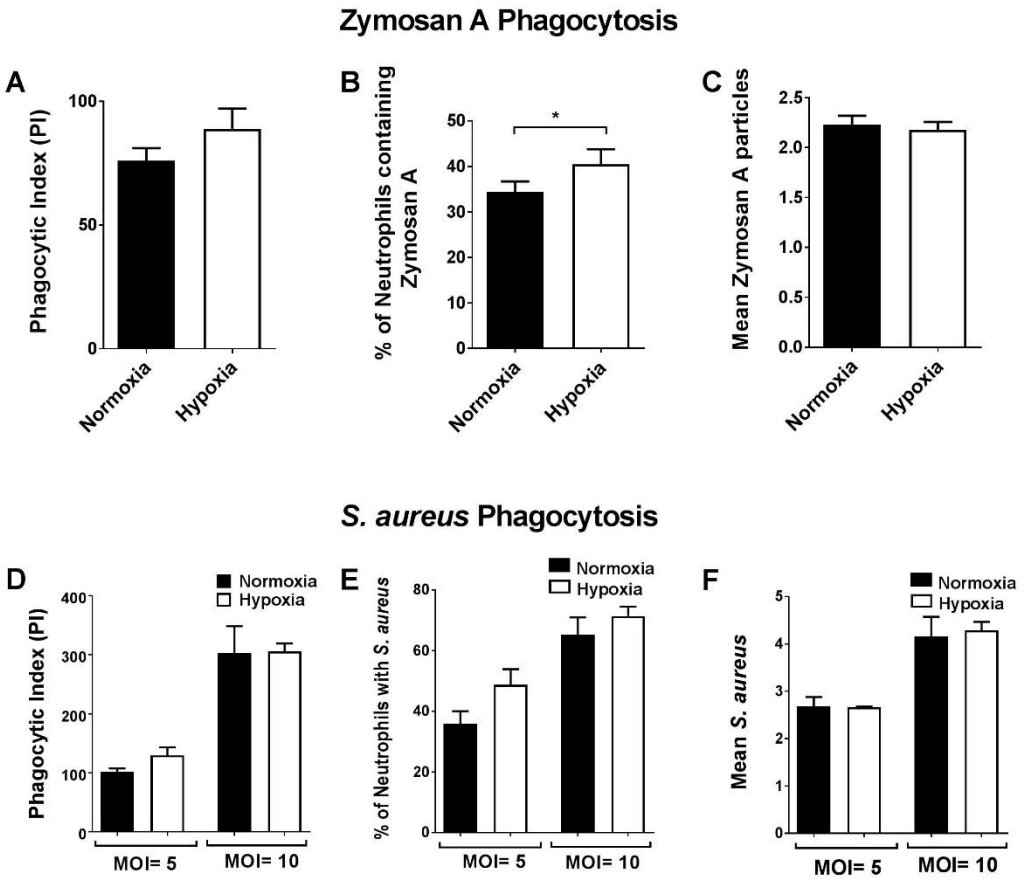


Figure 3.10. Effects of hypoxia on neutrophil phagocytosis. Neutrophils were cultured in normoxia and hypoxia for 4 hours followed by the addition of Zymosan A particles [0.2 mg/ml] for 15 minutes or heat-killed *S. aureus* [MOI 5 &10] for 30 minutes and then phagocytosis was assessed by light microscopy. (A, B &C) The Zymosan A phagocytosis ability was assessed in terms of phagocytic index, % of neutrophils containing Zymosan A and the mean zymosan A particle per neutrophil, respectively. (D, E, & F) The *S. aureus* phagocytosis ability was assessed in terms of phagocytic index, the percent neutrophils containing *S. aureus* and the mean bacteria per neutrophil, respectively. The phagocytic index was calculated by multiplying the percent of neutrophils containing the prey particles with the mean prey particles per neutrophil. Data represent three independent experiments with duplicate measurements under each condition, and the values are expressed as mean ± SEM. The closed and open bars represent normoxia and hypoxia, respectively. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significance was calculated by paired t-test (A-C) or one way ANOVA with Bonferroni correction (D-F).

3.3. Discussion

Acute exacerbations are a significant cause of morbidity and mortality in COPD patients (Donaldson et al., 2002). Bacterial and viral infections are demonstrated to be frequent triggers of the exacerbations (Sethi, 2011). Nearly 50% of COPD exacerbations are associated with bacterial infection such as *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Haemophilus parainfluenzae,* and *Pseudomonas aeruginosa,* and these bacterial species are noted to be key contributors in the disease pathophysiology (Sapey and Stockley, 2006). Despite increased accumulation of macrophages (Barnes, 2004) and neutrophils (Stockley, 2002) in the airway of COPD patients, bacterial persistence is a common feature, suggesting that neutrophils (and of macrophages) have developed a clearance defect that could reflect defects in phagocytosis, killing mechanisms, opsonisation or defects in other humoral components. Since there is evidence of phagocytosis defect of macrophages in COPD (Donnelly and Barnes, 2012)., neutrophil phagocytosis defect could contribute to this clearance defect in this disease settings. In this chapter, in order to explore a potential phagocytosis defect in COPD neutrophils, the phagocytic capacity was compared between circulatory neutrophils from COPD patients and healthy control subjects. A role for pro-inflammatory factors such as IFN-γ, mROS, and hypoxia in modifying neutrophil phagocytosis was also investigated. Circulatory neutrophils from COPD patients have a modest phagocytosis defect which was not been corrected with Sulforaphane. Furthermore, scavenging mROS, IFN-γ priming, and hypoxia did not affect neutrophil phagocytosis.

Extensive studies on the phagocytosis activity of monocytes and alveolar macrophages from COPD patients suggest that these cells are defective in phagocytosis (Donnelly and Barnes, 2012). However, few studies comparing neutrophil phagocytosis between COPD versus healthy subjects have been

performed and those that have yield inconsistent results (Fietta et al., 1988, Muns et al., 1995, Prieto et al., 2001, Walton, 2014). The modest phagocytosis defect observed in this thesis is consistent with previous literature where it was shown that cigarette smoke, an important etiological agent in COPD pathology, reduces neutrophil phagocytosis *in vitro* (Stringer et al., 2007) and also decreases *Pseudomonas aeruginosa* clearance in an *in vivo* mouse infection model (Drannik et al., 2004). Although some believe that the phagocytic defect observed in COPD neutrophils is acquired in the pro-inflammatory lung micro-environment, a range of literature implies that the circulatory neutrophils from COPD patients also demonstrate intrinsic functional defects (Burnett et al., 1987, Fietta et al., 1988, Milara et al., 2012, Noguera et al., 2001, Prieto et al., 2001, Sapey et al., 2011, Woolhouse et al., 2005).

The above-mentioned existing comparative studies of neutrophil phagocytosis between COPD and healthy subjects were performed in opsonic-dependent mode, however, it is thought that non-opsonic phagocytosis is the predominant mode of phagocytosis in the lung where phagocytic cells express comparatively lower levels of opsonic receptors (Stokes et al., 1998, Taylor et al., 2010). Therefore, I have used non-opsonic *S. aureus* in this study. In addition to this, neutrophils can phagocytose non-opsonic *S. aureus* efficiently (Lu et al., 2014), compared to other pulmonary pathogens such as *Streptococcus pneumoniae* (Gentry et al., 1995). In the *S. aureus* phagocytosis assay, 30 minutes cut off time was chosen for the incubation of the bacteria with neutrophils since neutrophils phagocytose *S. aureus* nearly to the saturation levels at this time point (Lu et al., 2014). The 30 minutes phagocytosis time period may not address the *in vivo* context, where the interaction time between neutrophils and the bacteria is thought to be much higher and also involves pathogen colonisation with multiple modes of infection. Since I compared the phagocytosis ability between COPD versus healthy subjects by adopting the traditional

phagocytosis assays using circulatory neutrophils and also used heat-killed *S. aureus*, the assay may not address the context of pulmonary infection *in vivo* adequately.

A limitation of my study is that the COPD patients and the healthy control subjects were not age-matched and overall, the healthy subjects were much younger individuals. Of note, the age of the healthy subjects could not be obtained as it was not allowed under ethical guidelines for these subjects. This is important because the phagocytic capacity of neutrophils is known to be depressed in elderly subjects due to immune senescence (Butcher et al., 2001, Chun-Hsiang Chiu 2011, Wenisch et al., 2000). Therefore, age-related impairments of phagocytic function in the COPD patients cannot be ruled out. A second limitation is that the blood samples for COPD patients (also healthy controls) used for the comparison were collected and subsequently travelled from Manchester to Sheffield, which took approximately one and half hours. This might cause an activation of neutrophils in the blood sample and subsequently might affect the phagocytosis phenotype studied. However, it would be possible to determine whether this travel time difference can impact on neutrophil functions by taking blood in Sheffield at the same time of blood collection at Manchester followed by comparing the effects on neutrophil activation and functions. In addition to these, the confounding effects of drugs such as inhaled steroids and statins on the impaired phagocytosis in the patients cannot be ruled out.

Oxidative Stress in the lung, as a result of long-term exposure to cigarette smoke or oxidised products of biomass fuels, has been suggested to be a major etiologic factor in the COPD pathogenicity (Sethi, 2011). Oxidative stress occurs as a result of an increased generation of ROS or a failure to scavenge ROS due to the impairment of cellular anti-oxidant defence system. Oxidative stress is thought to have a major role in the impairment of phagocytic function, as this is appeared to be a common mechanism in the suppression of surface receptor on phagocytes, for example, its effect on the reduced expression of MARCO in macrophages, which is

a scavenger receptor and aid in the phagocytosis process (Harvey et al., 2011). Therefore, the use of anti-oxidants may have the potential to reverse phagocytosis defects in a clinical settings. A body of evidence suggests that the use of anti-oxidants can ameliorate the phagocytosis defect observed in COPD macrophages. For example, the anti-oxidants N-acetyl cysteine and procysteine have been reported to improve phagocytosis and efferocytosis (respectively) by monocytes and macrophages from COPD patients (Hodge et al., 2011, Vecchiarelli et al., 1994). Furthermore, both N-acetyl cysteine and procysteine have been noted to improve the phagocytosis of *S. aureus* by alveolar macrophages using rat models of macrophage dysfunction (Brown et al., 2007). Another promising approach is the use of activators of the transcription factor Nrf2 that regulate over 200 genes of cellular anti-oxidants and detoxifying enzymes via binding to an anti-oxidant response element in the promoter region of these genes (Kobayashi and Yamamoto, 2006). A decline in the anti-oxidant capacity of Nrf2 has been reported in the lung of COPD patients (Malhotra et al., 2008), demonstrating an importance of this factor in a disease context. The Nrf2 activator, sulforaphane, has been shown to improve *H. influenzae* phagocytosis by macrophages from COPD patients via an increased expression of cell surface receptor MARCO (Harvey et al., 2011). In my study, however, sulforaphane was unable to increase phagocytosis of *S. aureus* by neutrophils from COPD patients. Thus this finding suggests that the dysregulated *S. aureus* phagocytosis by COPD neutrophils is not governed by ROS, or that the modest underlying defect in COPD is in fact not biologically significant and therefore not modifiable. Sulforaphane requires a significant length of time to reduce cellular ROS as its mode of action in reducing the ROS depends on transcription and translation of anti-oxidant genes (Kobayashi and Yamamoto, 2006). Although it was shown in this study that sulforaphane treatment for 4 hours reduces cellular ROS, however, it is possible that this treatment is not long enough to reduce ROS levels that have the ability to change the phagocytosis function in neutrophils. Therefore, a longer

treatment may show greater effects on phagocytosis. Considerably longer treatment may be impractical, however, because of rapid neutrophil constitutive apoptosis. In addition to these, sulforaphane has been noted to affect on cell viability (Kim et al., 2016, Royston et al., 2017, Suppipat et al., 2012), which could affect the result of phagocytosis; however, it could be addressed by using cell viability assays (e.g. MTT assay) following sulforaphane treatment.

Although ROS can be generated from a number of sources within cells, an important source is the mitochondrion. Though it has long been believed that mROS are merely by-products of cellular metabolism, mROS is currently being considered as having a key role in cellular signalling (Finkel, 2012). Dysregulated mROS production is associated with a number of pathological conditions including cancers and inflammatory diseases (Li et al., 2013, Naik and Dixit, 2011). In the context of lung inflammation, mROS is associated in driving inflammation and smooth muscle remodelling in patients with COPD (Wiegman et al., 2015). Furthermore, it has been noted that the phagocytic uptake and MARCO expression was reduced in macrophages when mROS was inhibited (Dehn et al., 2016), suggesting that mROS plays an important role in macrophage phagocytosis. In contrast, the phagocytosis of zymosan A or *S. aureus* by neutrophils was not affected when mROS was scavenged with mito-TEMPO, suggesting that neutrophil phagocytosis is not subjected to mROS-mediated regulation. It may be possible that mROS in neutrophils may function differentially than that of macrophages as neutrophils have very few and functionally different mitochondria that are dedicated to cell death signalling (Maianski et al., 2004).

Since mito-TEMPO is a ROS scavenger and its mode of action does not depend on the modulation of cell signalling or gene expression, the ability of this in effectively reducing mROS is expected within a shorter period of time. Thus, in the zymosan A phagocytosis assay, neutrophils were pre-incubated for one hour.

However, our group has shown that 4 hours pre-incubation with sulforaphane reduced mROS in neutrophils (discussed below) and therefore, I chose 4 hours time point for *S. aureus* phagocytosis. However, this longer time point could also be used for zymosan A phagocytosis for confirming the result of without effect of mROS on neutrophil phagocytosis.

The sulforaphane and mitochondrial ROS scavenger, mito-TEMPO both of which have been previously shown to generate a cellular anti-oxidant pool (Dikalova et al., 2010, Morimoto et al., 2006). The effectiveness of sulforaphane [100 nM] and mito-TEMPO [100 μM] in reducing the levels of mROS was confirmed by using the mitochondrial ROS indicator, mitosox in a flow cytometer based assay. Mitosox is a triphosphonium derivative of di hydroethidium (DHE) that enriches more than 100 fold within mitochondria than cytosol because of the mitochondrial negative membrane potential (Smith and Murphy, 2011). Sulforaphane and mito-TEMPO independently reduced the levels of mROS, suggesting that the concentrations of these two compounds used in this study were effective in reducing mROS production.

IFN-γ is a cytokine secreted predominantly by Th1 lymphocytes and plays a critical role in the immune response to pathogens (Schoenborn and Wilson, 2007). A range of literature has suggested that IFN-γ signalling is upregulated in the lung of COPD patients, particularly during acute exacerbations (Grumelli et al., 2004, Hens et al., 2008, Makris et al., 2008, Panzner et al., 2003, Reeves et al., 2010, Singh et al., 2010). The effect of IFN-γ is well characterised in macrophages, regulating the initiation of innate immune responses. In particular, IFN-γ priming in LPS-induced host-response in macrophage is important, where IFN-γ primes macrophages so that the macrophage responds very rapidly to LPS-mediated initiation of host-response, such as NF-kB activation, than the responses in the presence of LPS alone (Jurkovich et al., 1991, Kamijo et al., 1993, Lorsbach et al., 1993). Macrophages form heterogeneous populations including M1 and M2 phenotypes (Goerdt and Orfanos,

1999, Gordon, 2003, Mantovani et al., 2004) and a number of studies have demonstrated the involvement of their phenotypes in multiple pathological conditions and outcomes of the diseases (Murray and Wynn, 2011). Like macrophages neutrophils are heterogeneous (Beyrau et al., 2012, Silvestre-Roig et al., 2016), and an increasing body of evidence suggests that neutrophil subsets are implicated in multiple pathological conditions, including the pathogen driven systemic inflammation and cancers (Beyrau et al., 2012, Silvestre-Roig et al., 2016). Since IFN-γ/LPS have been noted to polarise macrophages to M1 pro-inflammatory phenotype (Gordon, 2003, Mantovani et al., 2004) IFN-γ/LPS were tested in neutrophils if these pro-inflammatory stimuli have the ability to shift neutrophils towards a phenotype with an altered ability in phagocytosis.

The concept of M1/M2 macrophage or N1/N2 neutrophil polarisation has been established by *in vitro* studies with artificial stimulators: some stimuli skew the polarisation to a certain direction whereas others have an effect to an opposite direction (Goerdt and Orfanos, 1999, Gordon, 2003, Ma et al., 2016, Mantovani et al., 2004). The situation may be much more complex *in vivo* contexts, where a wide number of stimuli is present, and therefore, may have near-infinite polarisation states rather than the bipolar states (Martinez and Gordon, 2014). In addition to this, the activation of these cells can also be influenced by a number of factors including, the presence of chemoattractants, the composition of the extracellular matrix and cell adhesion. Therefore, translating the *in vitro* result to mimic the conditions in diseases would be far more difficult (Davies et al., 2013).

Our group has shown that human neutrophils respond to IFN-γ priming and subsequent LPS treatment with an up-regulation of HIF-1α in neutrophils (unpublished data) and furthermore, the existing literature suggests that HIF-1α expression regulates macrophage phagocytosis and bactericidal capacity (Anand et al., 2007, Peyssonnaux et al., 2005). These considerations led me to hypothesise

that IFN-γ/LPS may regulate neutrophil phagocytosis and therefore, phagocytosis activity was assessed following IFN-γ/LPS treatment in terms of *in vitro* phagocytosis of Zymosan A, *E. coli* and clinically relevant pathogen *S. aureus.* However, IFN-γ/LPS treatment did not regulate the phagocytosis of Zymosan A, *E. coli or S. aureus.* One of the limitations in this experiment is that neutrophils were treated with IFN-γ only for 4 hours while considerably longer treatment times are described in the literature for macrophages, which is impractical for neutrophils as they undergo apoptosis in a time dependent manner. Again, in the zymosan A phagocytosis assay, variable data were obtained from donor to donor with an up-regulation in some donor and down-regulation with others, which may be explained by the fact that individual donor responds differently to IFN-γ, as is seen in the case of TNFα (Kisich et al., 2002). In addition, the counting of zymosan A and *S. aureus* particles can be subjective; therefore a flow cytometry assay was adopted to reproduce the finding.

Among the two conjugated probes used in the flow based *E. coli* phagocytosis assay, Alexafluor 488-*E. coli* may produce some signals even though the bacteria has not phagocytosed because the bacteria may adhere to the surface of the cells. For this reason, the background signals were subtracted by using 4^{o}C assay control. Alternatively, a relatively better fluorochrome conjugate called pHrodo green-*E. coli was* chosen which produces a signal only under an acidic condition which is attained only when the bacteria get internalized followed by the formation phagosome. Using 4 different phagocytosis assays, I have shown that the neutrophil phagocytosis was not affected by IFN-γ/LPS treatment.

Although IFN-γ/LPS did not have any effect on phagocytosis in neutrophils from healthy subjects, however, these assays could be repeated with neutrophils from COPD patients, which might show altered phagocytosis phenotype and thus require further studies to address this.

In this study, the effect of hypoxia was also examined in phagocytosis assays, since hypoxia may represent to an environmental cue to inflammatory sites, for example during airway inflammation, which is characteristically hypoxic due to the changes of normal lung structure leading to a restriction of airflow to the inflamed tissues (Han and Mallampalli, 2015, Hoenderdos and Condliffe, 2013). In the phagocytosis assay with Zymosan A particles although a short period of hypoxia (1 hour) did not affect the phagocytosis (data not shown), four hours hypoxia significantly enhanced the percent phagocytosis but not the phagocytic index or the mean particles per cell**,** suggesting that hypoxia affects on the shifting the neutrophils towards an 'ingester' phenotype.; whereas the phagocytic index was unaffected in response to hypoxia. This may be explained by the fact that a relatively higher number of neutrophils shifted towards the 'ingester' phenotype that had to compete for the same number of zymosan A particles and therefore could be a reason for decreasing the value of the mean zymosan A particles that confer the phagocytic index unaffected. Or the small differences of the percent phagocytosis in the response of hypoxia (34.38±3.26 to 40.54±3.26) may not be biologically significant.

In conclusion, COPD neutrophils had a modest *S. aureus* phagocytosis defect; however, it was not strictly confirmed as it can be affected by the ages of study subjects. Furthermore, the phagocytosis capacity was not changed by sulforaphane treatment. I found no evidence that the neutrophil phagocytosis process was related to mROS-, IFN-γ/LPS- or hypoxia-driven regulation, but further exploration of the mechanisms is required to definitively rule out a role for these factors and to investigate other potential mechanisms.

Chapter 4: A protein kinase inhibitor library screen identified kinases that may regulate neutrophil apoptosis.

4.1. Brief Introduction

Chronic inflammatory diseases such as COPD are a major cause of ill health (WHO, 2013), and a broad range of the literature suggests that persistent neutrophilic inflammation due to a neutrophil apoptosis defect may contribute to chronic inflammatory diseases such as COPD (Hoenderdos and Condliffe, 2013, Zhang et al., 2012). There are no existing treatment strategies that have the potential to reverse the cell-mediated lung destruction observed in COPD. A major barrier to the design of anti-inflammatory therapies targeting neutrophil apoptosis is a lack of complete understanding in the mechanisms regulating neutrophil apoptosis, and therefore, identifying novel and therapeutically targetable neutrophil apoptosis pathway(s) is an unmet need to design effective therapy for treating chronic inflammation. Protein kinases comprise the largest family of evolutionarily related proteins, and 518 distinct kinases encompass 1.7% of the human genome (Manning et al., 2002). Protein kinases mediate transient protein phosphorylation at specific amino acid residues in 30% of all human proteins and play crucial roles in cellular processes, including cell signalling, metabolism, cell survival, and apoptosis (Hubbard and Cohen, 1993). Genotyping and phenotyping studies suggest that aberrant activity of protein kinases are associated with >400 human diseases (Melnikova and Golden, 2004), and to date, the US Food and Drug Administration (FDA) has approved over 25 protein kinase inhibitor based drugs and there are hundreds of further potential candidates for clinical evaluation (Bamborough, 2012, Cohen and Alessi, 2013, Wu et al., 2015). A broad range of literature highlights the importance of kinases in the regulation of neutrophil apoptosis (Burgon et al., 2014, Juss et al., 2012, Rossi et al., 2006, Wang et al., 2003, Webb et al., 2000), and promoting neutrophil apoptosis has been

reported to enhance inflammation resolution in multiple *in vivo* experimental models (Burgon et al., 2014, Chello et al., 2007, Heasman et al., 2003, Ren et al., 2008, Rossi et al., 2006). In this chapter, neutrophil apoptosis in COPD and healthy control subjects was studied to identify potential apoptosis defects in neutrophils from COPD patients. Also, using a previously published and open-sourced Published Kinase Inhibitor Sets (PKIS), a pharmacological screening approach was adopted to identify novel protein kinases that may play an important role in regulating neutrophil survival.

4.2. Results

4.2.1. COPD neutrophils are more resistant to pyocyanin-induced apoptosis compared to neutrophils from healthy subjects.

Neutrophilic inflammation in the airway is a prominent feature in COPD pathology. Increased number of neutrophils in the airway correlates with a deterioration of lung function (Stanescu et al., 1996), and also neutrophilic inflammation is a feature of COPD exacerbations (Gompertz et al., 2001). An increasing body of literature implies that COPD circulatory neutrophils differ phenotypically compared to healthy neutrophils (Burnett et al., 1987, Milara et al., 2012, Noguera et al., 2001, Sapey et al., 2011). To identify any potential neutrophil apoptosis defects in COPD, neutrophils were isolated from COPD patients and healthy control subjects and neutrophil apoptosis was assessed by light microscopy at 6 and 20 hours following culture with or without GMCSF [50 U/ml] (Renshaw et al., 2003) or pyocyanin [50 µM] (Usher et al., 2002). Demographic characteristics of COPD patients (obtained from the Manchester Cohort, COPD MAP) are presented in Chapter 3 in **table 3.1.** GMCSF is a known neutrophil survival stimulus (Klein et al., 2000, Kobayashi et al., 2005), whereas pyocyanin is a pseudomonas toxin which has been shown to induce neutrophil apoptosis *in vitro* (Usher et al., 2002). Neutrophil apoptosis in the presence of pyocyanin is significantly greater in healthy control subjects compared to COPD

neutrophils at 6 hours (p=0.006), whereas similar apoptosis rates were observed following treatment with GMCSF at this timepoint (p=0.32) (**Figure 4.1 A**). The apoptosis rates were also found to be similar in COPD and healthy control subjects at 20 hours following GMCSF treatment (p>0.99) (**Figure 4.1 B**). The constitutive rates of apoptosis observed at both of the timepoints were also similar between COPD versus healthy control subjects (p=0.56 and p>0.99 for 6 and 20 hours, respectively) (**Figure 4.1 A&B**).

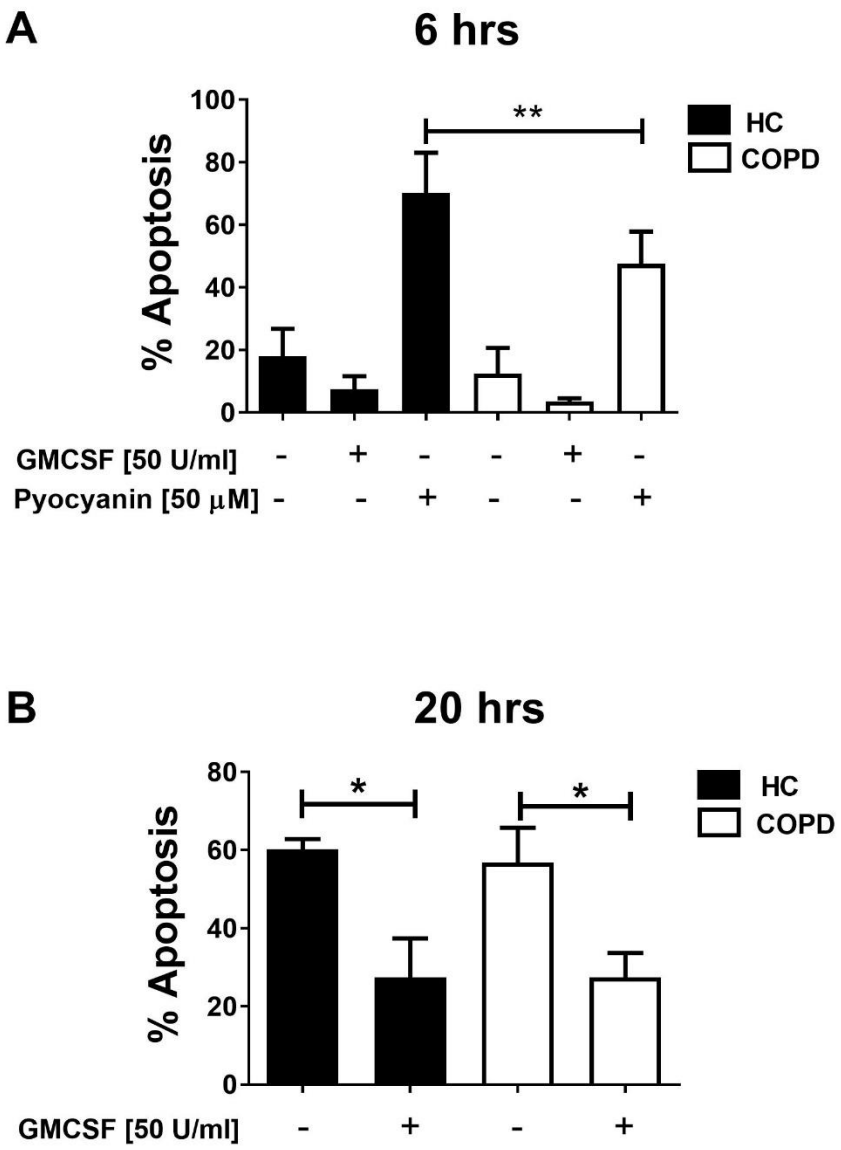


Figure 4.1. COPD neutrophils are more resistant to pyocyanin-induced apoptosis compared to healthy neutrophils. (A) COPD and healthy control (HC) neutrophils were cultured with or without GM-CSF [50 U/ml] or pyocyanin [50 μM] for 6 hours [n=6 (COPD) 5 (HC)] followed by an assessment of apoptosis by light microscopy. (B) Neutrophils from COPD and HC were co-cultured with GMCSF for 20 hours [n=6 (COPD) 5 (HC)] followed by the assessment of apoptosis by light microscopy. Data are expressed as mean percent apoptosis ± SEM with duplicate measurements under each condition. A total of 300 neutrophils were counted for each measurement by light microscopy. The closed and open bars represent HC and COPD, respectively. Statistical significance was calculated by one way ANOVA (with Bonferroni's post-test), and expressed as *p≤0.05, **p≤0.01.

4.2.2. Screening a kinase inhibitor library in neutrophil apoptosis assays

Targeting apoptosis pathways is considered as an attractive choice for designing anti-inflammatory therapies for chronic inflammatory diseases (El Kebir and Filep, 2013b). Kinases are increasingly implicated in diseases, altering cellular phenotypes (Lahiry et al., 2010), and kinase inhibitors are being considered as a growing class of therapeutics (Wu et al., 2015). To identify protein kinase regulated survival pathways which may ultimately be targeted for inflammation resolution, a library of protein kinase inhibitors was screened in a human neutrophil apoptosis assay.

4.2.3. Protein kinase inhibitor compound library

In this study, I used a library called PKIS consisting of 367 inhibitor compounds which has been published and made available to researchers around the world by the GlaxoSmithKline (GSK) (Drewry et al., 2014) and obtained in collaboration with Professor Steve Renshaw, University of Sheffield. All the compounds in this library are well known and are published in the scientific literature (Drewry et al., 2014). This inhibitor set has been profiled for its selectivity against 224 recombinant human protein kinases classified as 196 protein kinases, 21 mutant protein kinases associated with disease and 5 lipid kinases. The activity profile of these inhibitor compounds for each of the 224 kinases was generated by Nanosyn Microfluidics Enzyme Assay (Elkins et al., 2016). Because of unavailability/technical failures with some inhibitors, out of the 367 inhibitors in the set 298 were screened in a high-throughput *in vitro* neutrophil apoptosis flow cytometry assay based on an acceleration of neutrophil apoptosis. Apoptosis was assessed by phosphatidyl serine (PS) exposure on the outer surface of apoptotic cells as measured by annexin-V staining. The PS exists on the internal surface of the plasma membrane of healthy cells, but irreversibly translocates to the outer surface of the plasma membrane during apoptosis (van Engeland et al., 1998). This was used in conjunction with Topro-3,

which is a DNA binding dye and readily permeable by necrotic cells, but not by healthy or early apoptotic cells.

4.2.4. Optimization of kinase inhibitor compound screen

Screening the library at an initial concentration of 62 µM would achieve a DMSO concentration of 2.5%. The effect of 2.5% DMSO on neutrophil apoptosis was therefore tested prior to screening. **Figure 4.2** A & B shows 2.5% DMSO does not alter neutrophil apoptosis compared to media control at 6 hours, as assessed by both flow cytometry (p=0.37, n=5) and light microscopy (n=2) (**Figure: 4.2 A&B**). High-throughput assessment of neutrophil apoptosis during screening would be carried out flow cytometrically by the Attune Auto Sampler, based on Annexin-V/Topro-3 positivity. This is a robotic acquisition process and acquires samples well by well, beginning from the 1st (A1) to the last well (H12) of the 96 well plate. The estimated time required for this is 90 minutes as per the pre-set program, but the Attune flow cytometer does not have any temperature control. Therefore, to ascertain whether the changes in apoptosis observed are not because of the time taken to acquire samples, a 96-well plate of neutrophils was cultured with media for 6 hours, stained with annexin-V/Topro-3 and acquired by the Attune flow cytometer, choosing wells in a random order across the entire plate. Apoptosis did not differ between wells and the average percent of apoptosis was 16.8 ± 0.18, ranging from 13.8 to 20.1 **(Figure 4.2 C).**

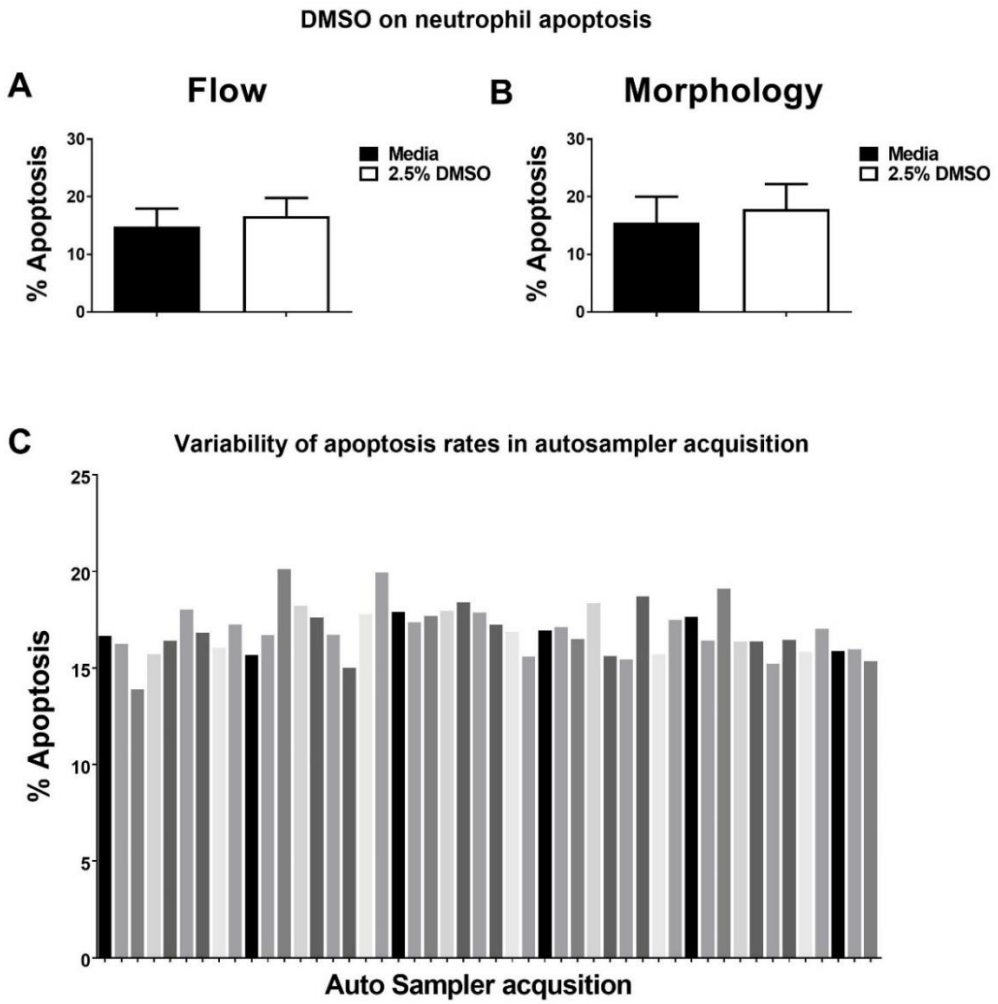


Figure 4.2. Optimization of primary screening. (A & B) Prior to performing the screen, neutrophils were cultured with or without 2.5% DMSO for 6 hours followed by the assessment of apoptosis by flow cytometry via annexin-V/Topro-3 staining (A, n=5) and by light microscopy (B, n=2). The apoptosis assessed by flow cytometry was calculated by the summation of annexin-V single positive and the annexin-V/Topro-3 double positive events. Data are expressed as mean percent apoptosis ± SEM with duplicate measurements under each condition. Media is the negative control for the assay. A total of 300 neutrophils were counted for each measurement for the assessment of apoptosis by light micrscopy. Statistical significance was calculated by paired t-test. (C) To ascertain whether the changes in apoptosis were not because of the time taken to acquire samples in the Attune auto sampler, neutrophils were cultured in random wells, beginning from the 1st (A1) to the last (H12) well of a 96 well plate, for 6 hours followed by staining with annexin-V/Topro-3, and the acquisition was performed by the Attune auto sampler. Data represent single experiment and expressed as percent of apoptosis.

4.2.5. Primary screening of kinase inhibitor compounds

In the primary screen round, 298 kinase inhibitors [62 µM] were tested by co-culturing these compounds with freshly isolated neutrophils for 6 hours, and apoptosis measured by annexin V/Topro-3. In this assay, GMCSF [50 U/ml] (Renshaw et al., 2003) and pyocyanin [50 µM] (Usher et al., 2002) were used as negative and positive controls, respectively, to ascertain that neutrophil apoptosis was predictably modifiable. Flow cytometry gating strategy for the assessment of neutrophil apoptosis is shown in **Figure 4.3**. Neutrophils were gated based on side (SSC) and forward scatter (FSC) properties (**Figure 4.3 A**), and apoptosis assessed by the annexin-V positive events to include both annexin-V single positive and the annexin-V/Topro-3 double positive events (**Figure 4.3 B**). Of note, the annexin-V single positive events indicate early apoptotic cells, whereas the annexin-V/Topro-3 double positive events indicate late apoptotic cells which may also contain necrotic population (Vermes et al., 1995). It is very difficult to distinguish between late apoptotic cells and necrotic cells. As it is difficult to test 298 compounds in a single assay, these compounds were tested in 5 healthy subjects across 5 individual experiments (80, 80, 63, 40 and 35 compounds tested separately in individual experiments). All screen data from all five assays are shown in **Figure 4.4**. In each of the assays, the negative control GMCSF reduced whereas the positive control pyocyanin enhanced neutrophil apoptosis, suggesting that neutrophils behaved as predicted with respect to apoptosis/survival pathways (**Figure 4.4**). Of note, to avoid bias, all kinase inhibitor compounds were run blindly during experimentation, meaning the identity of the inhibitors was not known until the data were collected and analysed. The apoptosis data in each of the individual screen assays were normalized by calculating the fold changes of apoptosis compared to DMSO control, and 62 compounds were found to accelerate neutrophil apoptosis ≥ 2-fold compared to DMSO control (**Figure 4.5 and Appendix**

7.6). The kinase targets of these compounds were identified from the kinase activity profiling data as described previously (Elkins et al., 2016) (**Figure 4.6**).

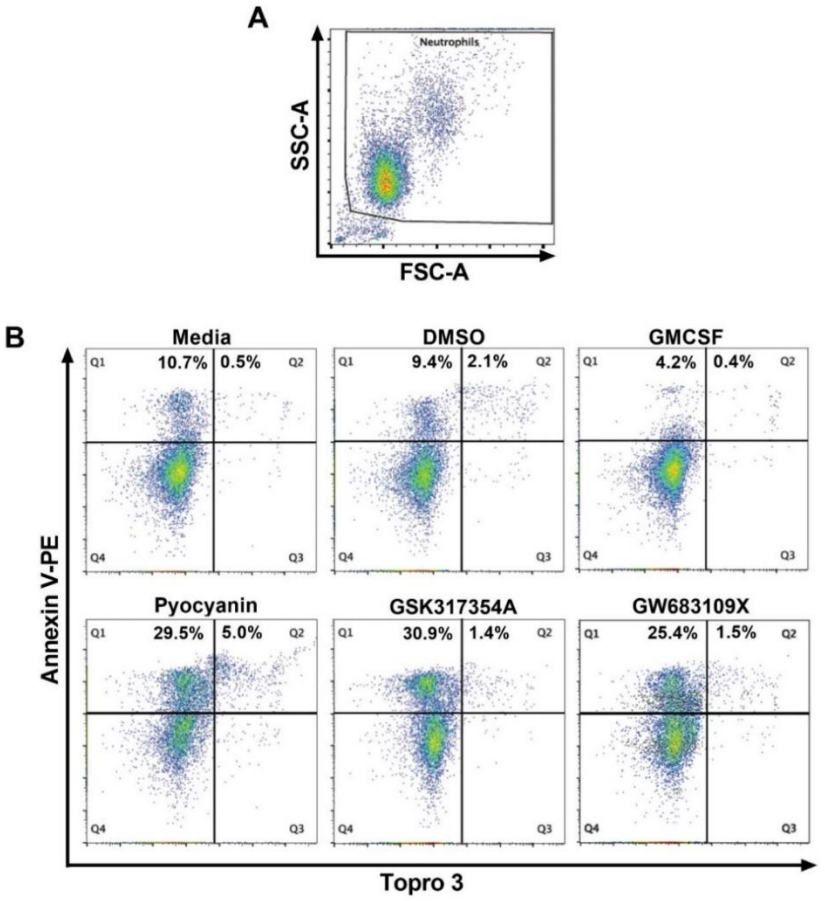
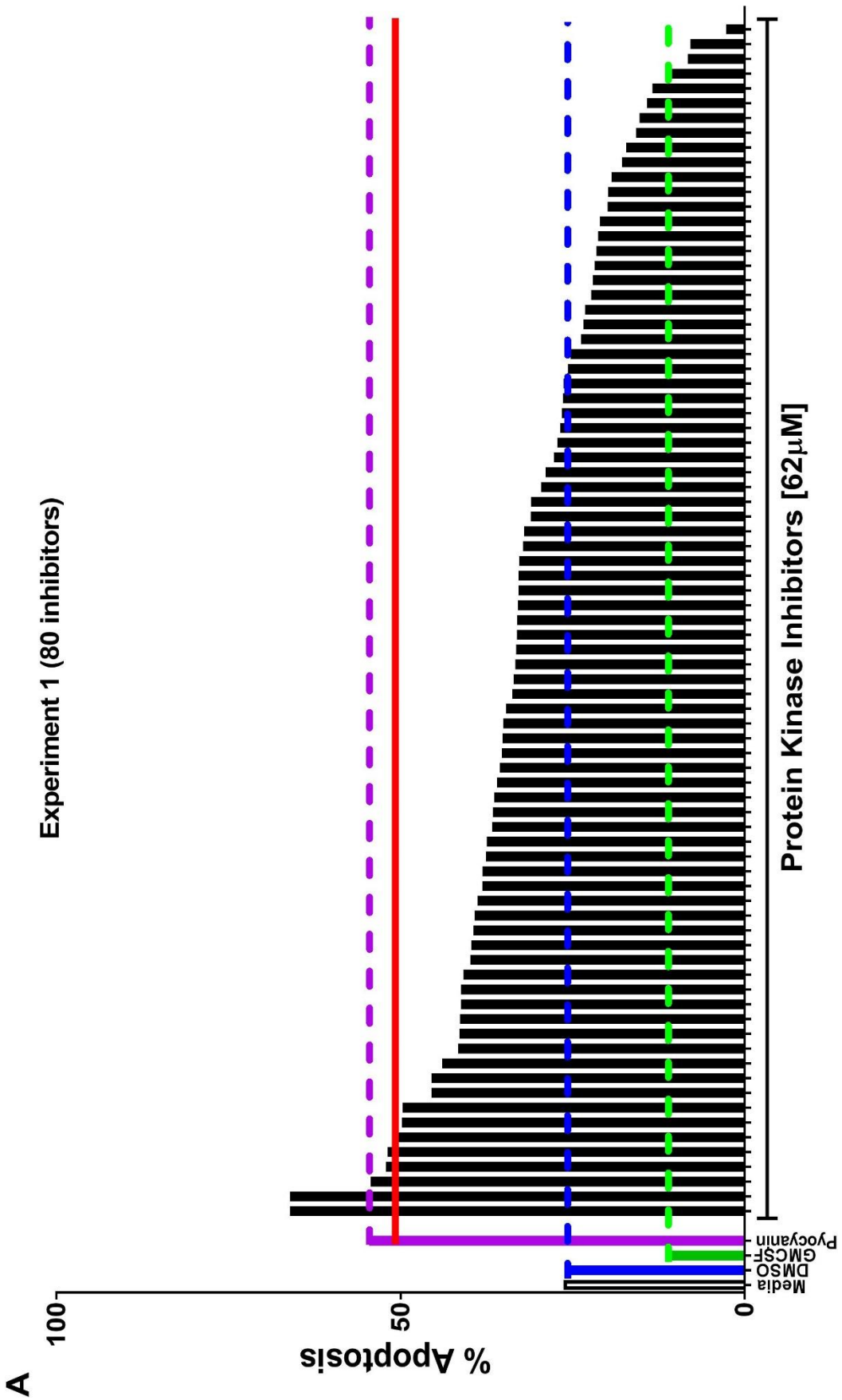
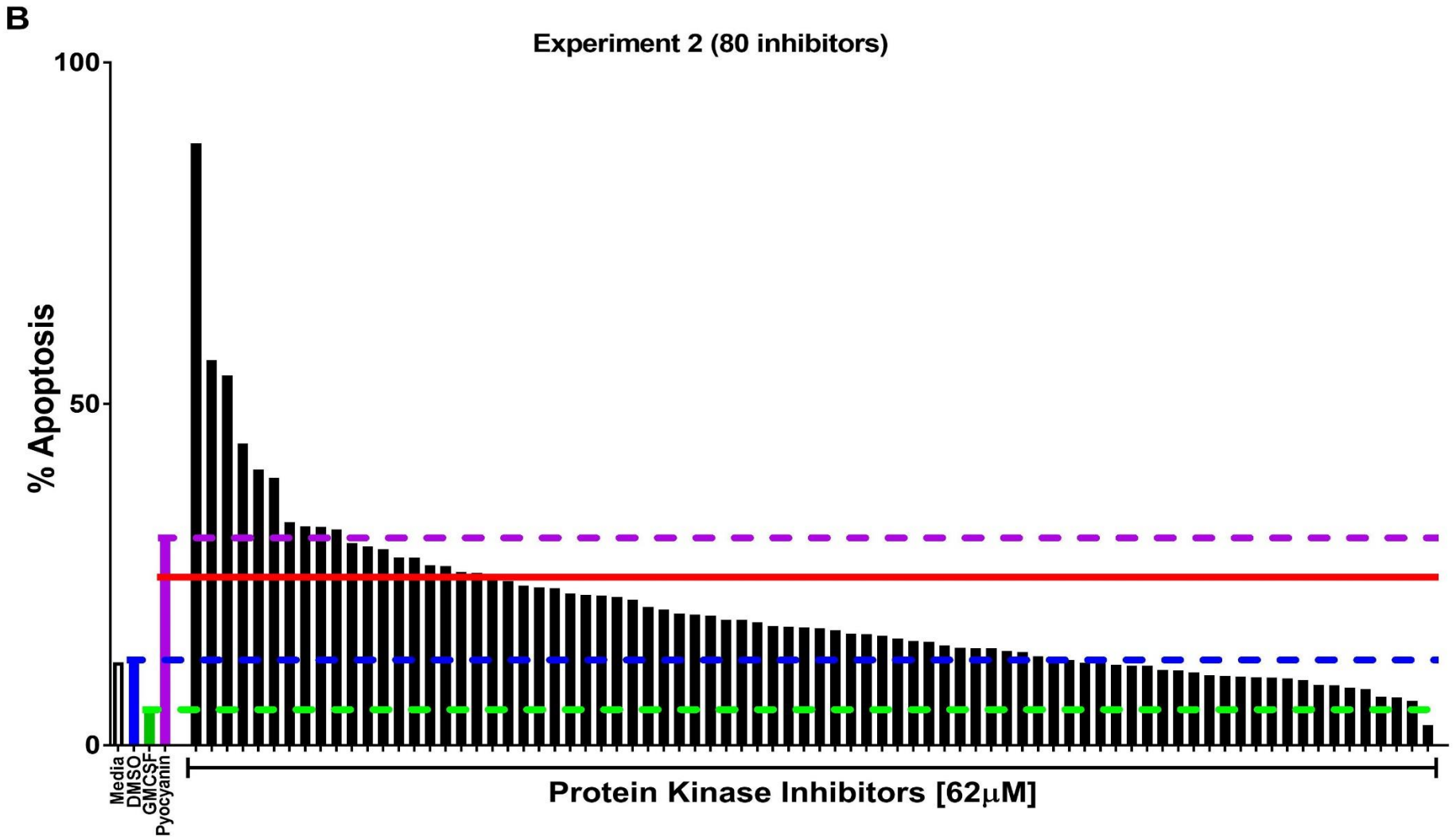
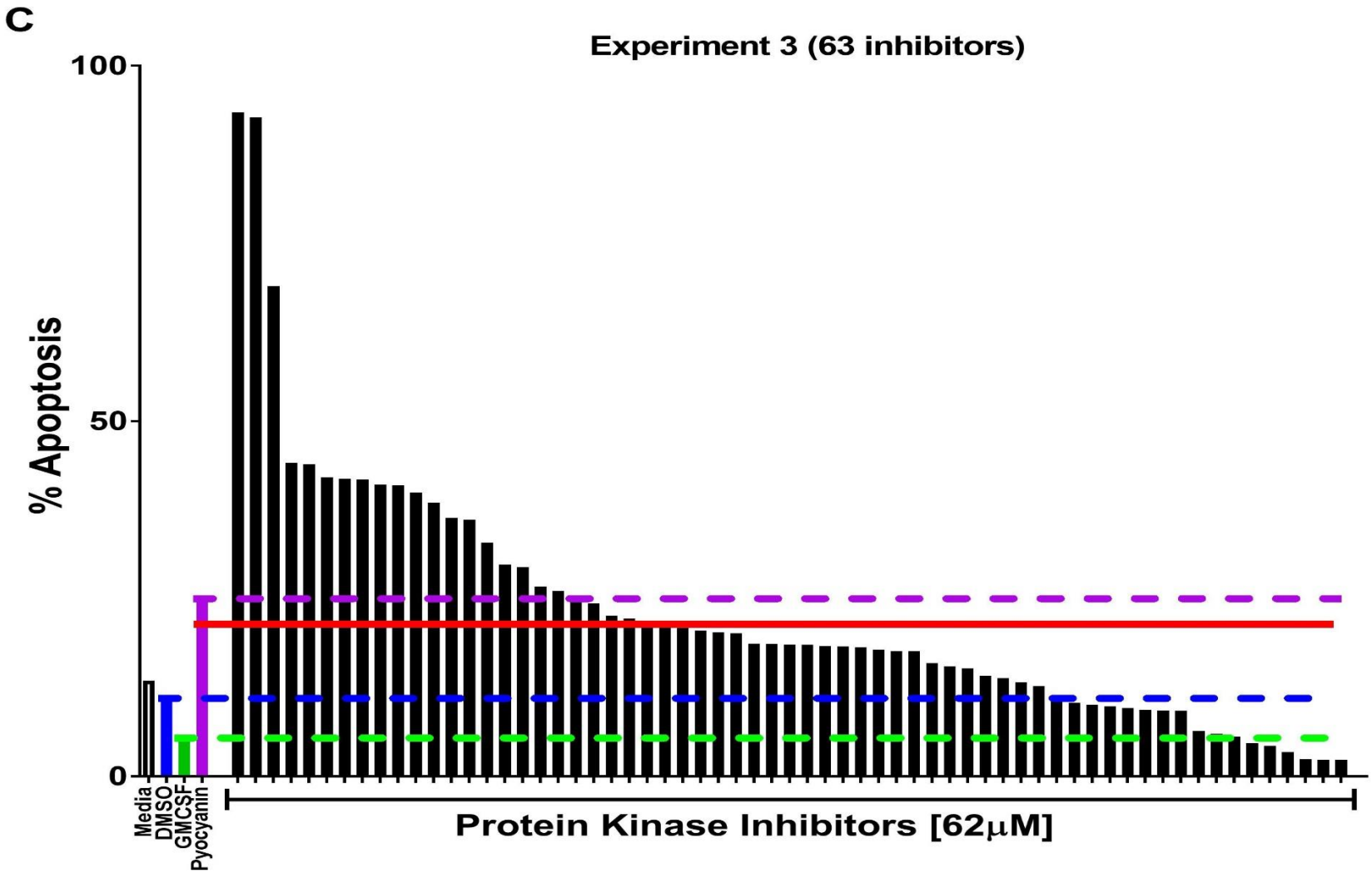


Figure 4.3. Flow cytometry gating strategy for the assessment of neutrophil apoptosis in the PKIS screening. Neutrophils from healthy subjects were cultured in the presence or absence of a Published Kinase Inhibitor Sets [PKIS, 62 µM], GMCSF [50 U/ml] and Pyocyanin [50 µM] for 6 hours followed by staining with annexin V-PE/Topro-3 for a flow cytometry assisted assessment of apoptosis. The acquisition of the samples was performed by the Auto sampler of the Attune flow cytometer. (A) Neutrophils were first gated based on the side scatter (SSC-A) and forward scatter (FSC-A) properties, and the SSC-A versus FSC-A distribution is shown in a pseudo-colour dot plot. (B) Gated neutrophils were then analysed based on the annexin-V versus Topro-3 distribution profile, and the apoptosis was calculated by the summation of annexin-V single positive and annexin-V/Topro-3 double positive events. Representative examples showing the distribution of annexin-V/Topro-3 in media, DMSO, GMCSF, pyocyanin and two inhibitor compounds (GSK317354A and GW683109X) that accelerated apoptosis ≥ 2-fold over control are shown. Media and DMSO is the negative control and the vehicle control for inhibitor compounds, respectively.


A
Experiment 1 (80 inhibitors)
% Apoptosis
100
50
0
Media
DMSO
GMCSF
Pyocyanin
Protein Kinase Inhibitors [62μM]


B
Experiment 2 (80 inhibitors)
100
50
0
% Apoptosis
Media
DMSO
GMCSF
Pyocyanin
Protein Kinase Inhibitors [62μM]


C
Experiment 3 (63 inhibitors)
100
50
0
% Apoptosis
Media
DMSO
GMCSF
Pyocyanin
Protein Kinase Inhibitors [62μM]

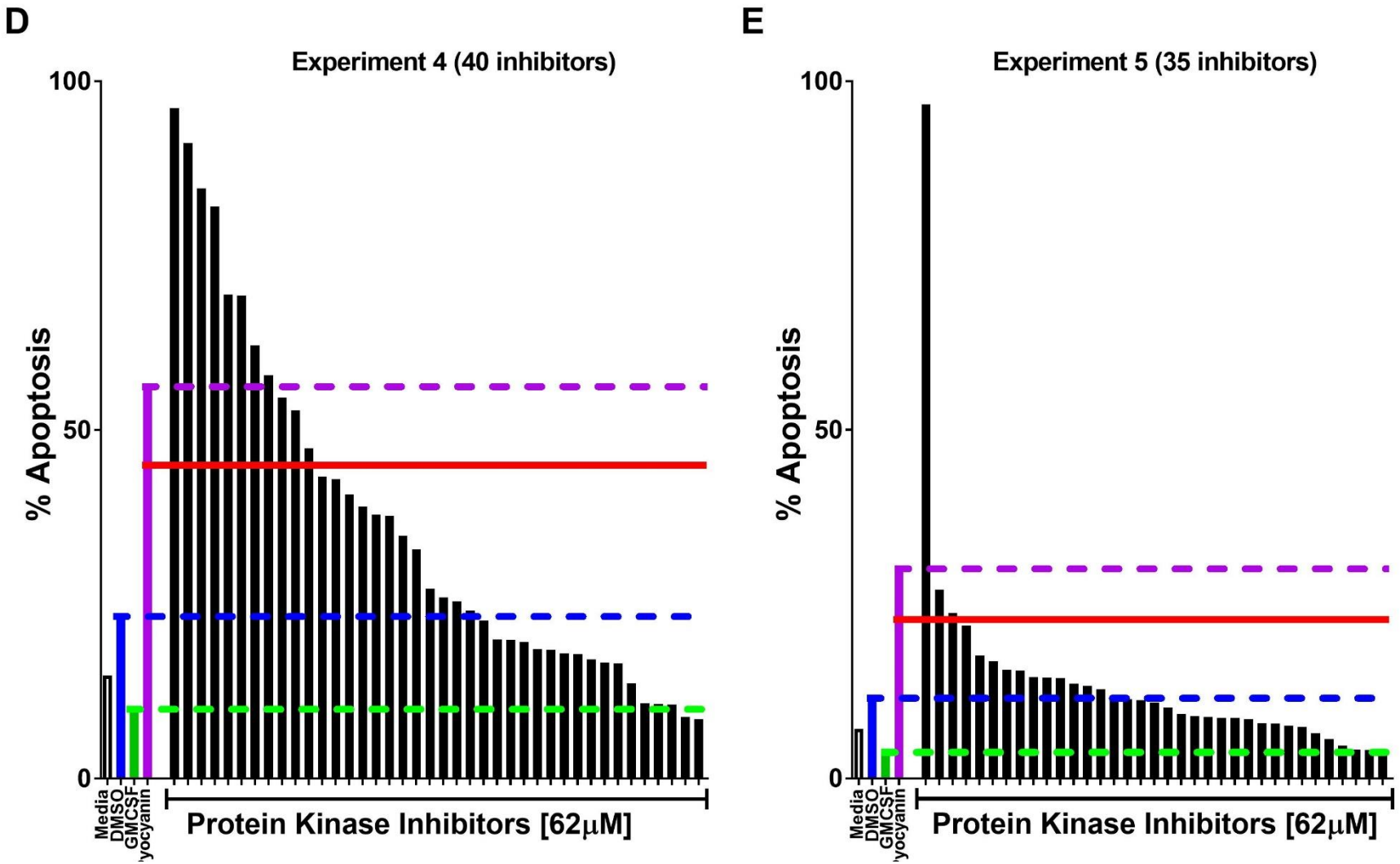

D
Experiment 4 (40 inhibitors)
100
50
0
% Apoptosis
Media
DMSO
GMCSF
Protein Kinase Inhibitors [62μM]
E
Experiment 5 (35 inhibitors)
100
50
0
% Apoptosis
Media
DMSO
GMCSF
Protein Kinase Inhibitors [62μM]

Figure 4.4. Primary screening of the PKIS in neutrophil apoptosis assay. In the primary screen round, 298 inhibitor compounds of the PKIS library were tested by co-culturing these compounds with neutrophils from healthy subjects for 6 hours followed by an assessment of neutrophil apoptosis by flow cytometry (as discussed in Figure 4.3). All the compounds [62 µM] were tested once using 5 healthy donors across 5 separate experiments [80 (A, Expt 1), 80 (B, Expt 2), 63 (C, Expt 3), 40 (D, Expt 4) and 35 (E, Expt 5) compounds tested separately in 5 individual experiments, respectively]. In every assay, GMCSF [50 U/ml] and pyocyanin [50 µM] were used as negative and positive controls, respectively, and coloured lines show the % apoptosis for these stimuli in relation to the inhibitor compounds. The data are expressed as the percent neutrophil apoptosis. The apoptosis rates in DMSO, GMCSF and pyocyanin were denoted by the dotted blue, green and purple lines, respectively. Media is the negative control; and DMSO is the vehicle control for inhibitor compounds. The solid red line denoted as the 2-fold change of apoptosis compared to DMSO control.

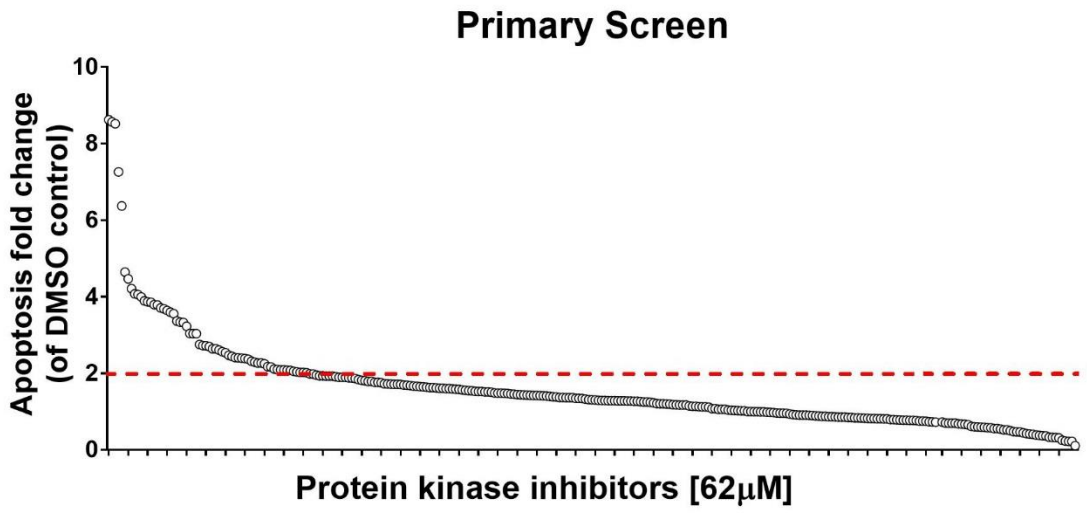


Figure 4.5. Primary screen data normalization. Primary screen data from each of the 5 individual assays were normalized by measuring the fold changes of apoptosis compared to DMSO (vehicle) controls and compiled into a single figure. The fold changes of apoptosis for all 298 compounds were then graphically presented in descending order where each of the open circle represents a single kinase inhibitor. Sixty two compounds were found to accelerate apoptosis ≥ 2-fold over control [as denoted by the red dotted line].

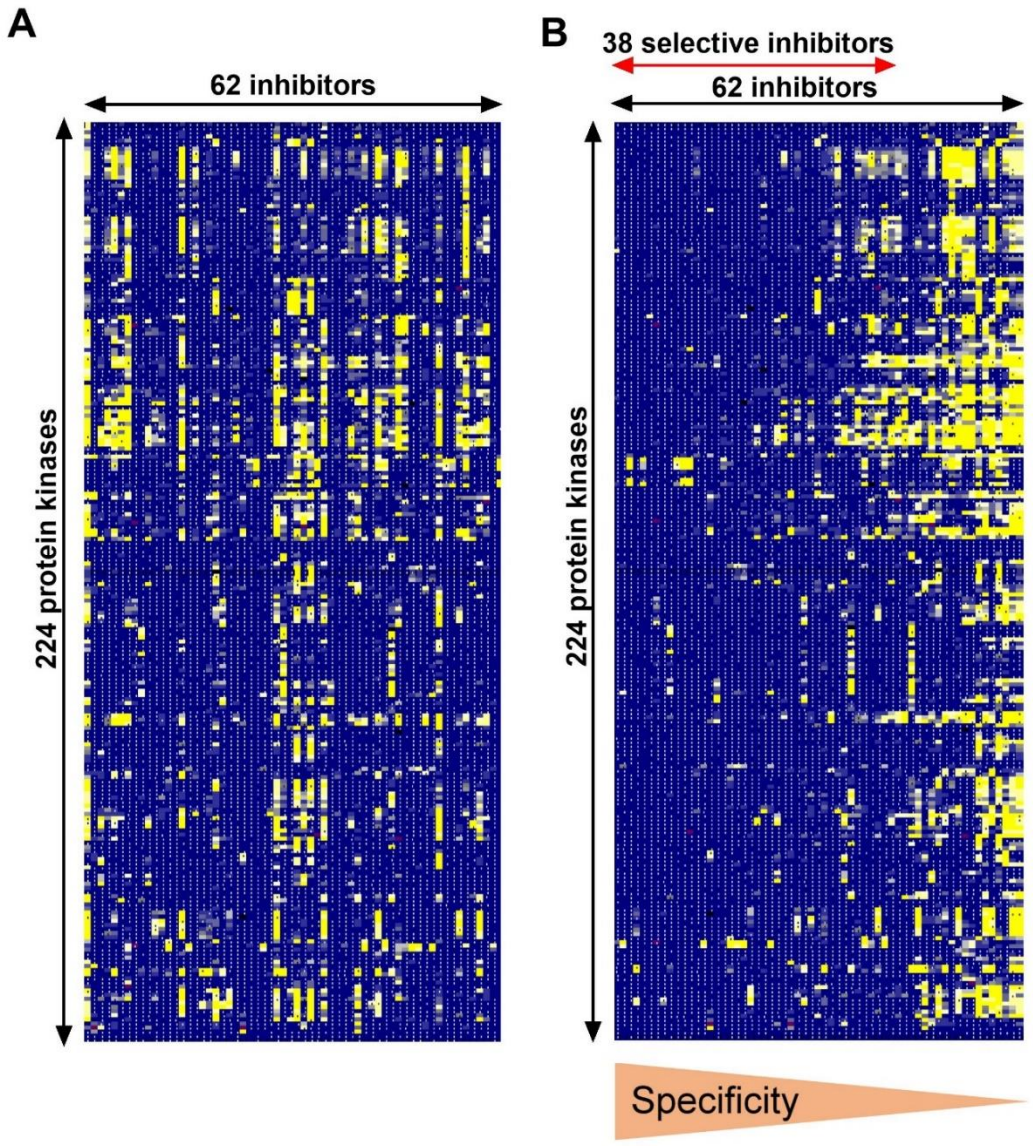


Figure 4.6. The kinase specificity profile of the inhibitor hit from primary screen. The kinase specificity of the PKIS has already been profiled against 224 human kinases as described previously (Elkins et al., 2016). (A) A heat map showing the kinase specificity profile of the 62 compounds identified in the primary screen. Each of the columns of the heat map represents each of the 62 inhibitors and the rows represent 224 kinases. The gradient of the yellow to blue colour represents high (100%) to no (0%) inhibitory capacities. (B) The same heat map in which the compounds were sorted in a way where the compounds to the left are most specific while the compounds to the right are broadly specific. Of the 62 inhibitors, 38 inhibitors that have higher specificity for kinase targets were chosen for secondary screening.

4.2.6. Secondary screening of kinase inhibitor compounds

In a secondary screening round, 38 compounds (from the 62 compounds identified in the primary screen) identified as having relatively higher specificity for their kinase targets were chosen based on the previously published specificity profile information of these compounds (**Figure 4.6**). These 38 compounds were screened at a concentration of 10 µM in 3 independent apoptosis assays by Annexin-V/Topro-3 staining as described for the primary screening. In the secondary screening round, the concentration of the inhibitors was reduced to 10 µM in order to minimise the potential false-positive results. As in the primary screen, GMCSF and Pyocyanin were also included in the assay and these stimuli resulted in reduced and enhanced apoptosis, respectively (**Figure 4.8**). For further validation of the primary screen result, along with these 38 compounds, 10 compounds that had no effect on apoptosis in the primary screening were also included. Only 1 out of the 10 compounds, used for validation of primary screening, accelerated neutrophil apoptosis (**Figure 4.7**). Secondary screening of the 38 compounds resulted in 11 compounds that robustly accelerated neutrophil apoptosis ≥ 2-fold compared to control as indicated by the green dashed line (**Figure 4.8**). Based on the specificity profile of these compounds (Elkins et al., 2016), a number of kinases inhibited by the 11 compounds greater than 50% were identified, including DYRK1B (Dual specificity tyrosine-phosphorylation-regulated kinase 1B), ErbB1, 2 & 4, KIT, PDGFR (Platelet-derived growth factor receptor), p38-MAPK and CDK6 (inset table in **Figure 4.8**). The compound that had the greatest pro-apoptotic effect was found to be highly specific for DYRK1B (inset table in **Figure 4.8**). Among the kinase targets identified, PDGFR was the most frequent target but was targeted by relatively non-selective inhibitors, while the ErbB family of RTKs (receptor tyrosine kinases) was the second most frequent target, which was targeted by 3 highly selective inhibitors (inset table in **Figure 4.8**). From the PKIS profile data published by Elkin et al (Elkins et al., 2016), the selectivity and

inhibitory profile of the 3 inhibitors specific for ErbB RTKs are shown in **Figure 4.9**. The dot plots showing the distribution of annexin-V and Topro-3 events for controls and the 11 compounds that resulted in apoptosis ≥ 2-fold are shown in **Figure 4.10**. Of note, as described for primary screen, the apoptosis rates were determined by the summation of the annexin-V single positive events with the annexin V/Topro-3 double positive events. The neutrophil death observed with these compounds was solely because of undergoing apoptosis rather than secondary necrosis, as these compounds did not markedly alter Topro-3 positivity (**Figure 4.10**).

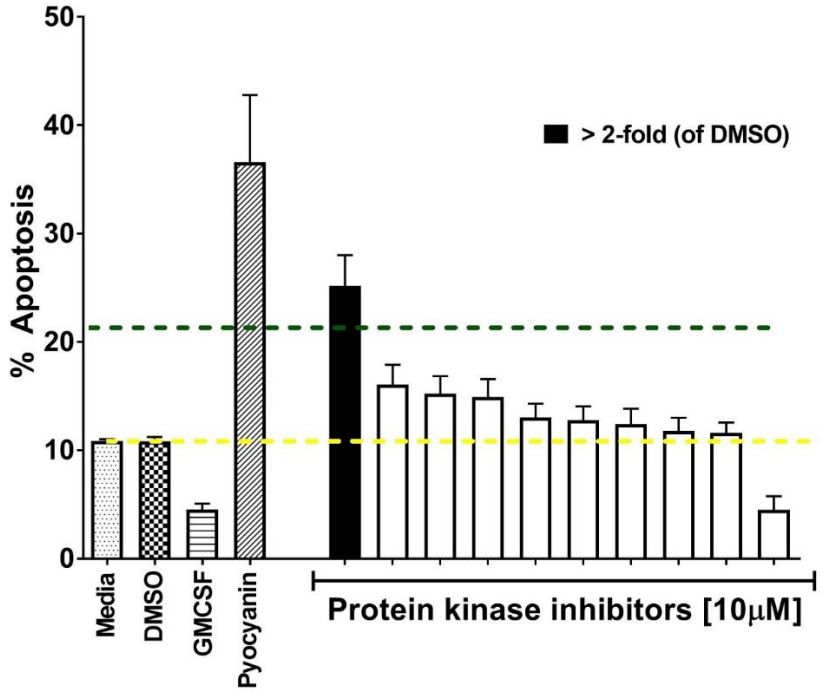
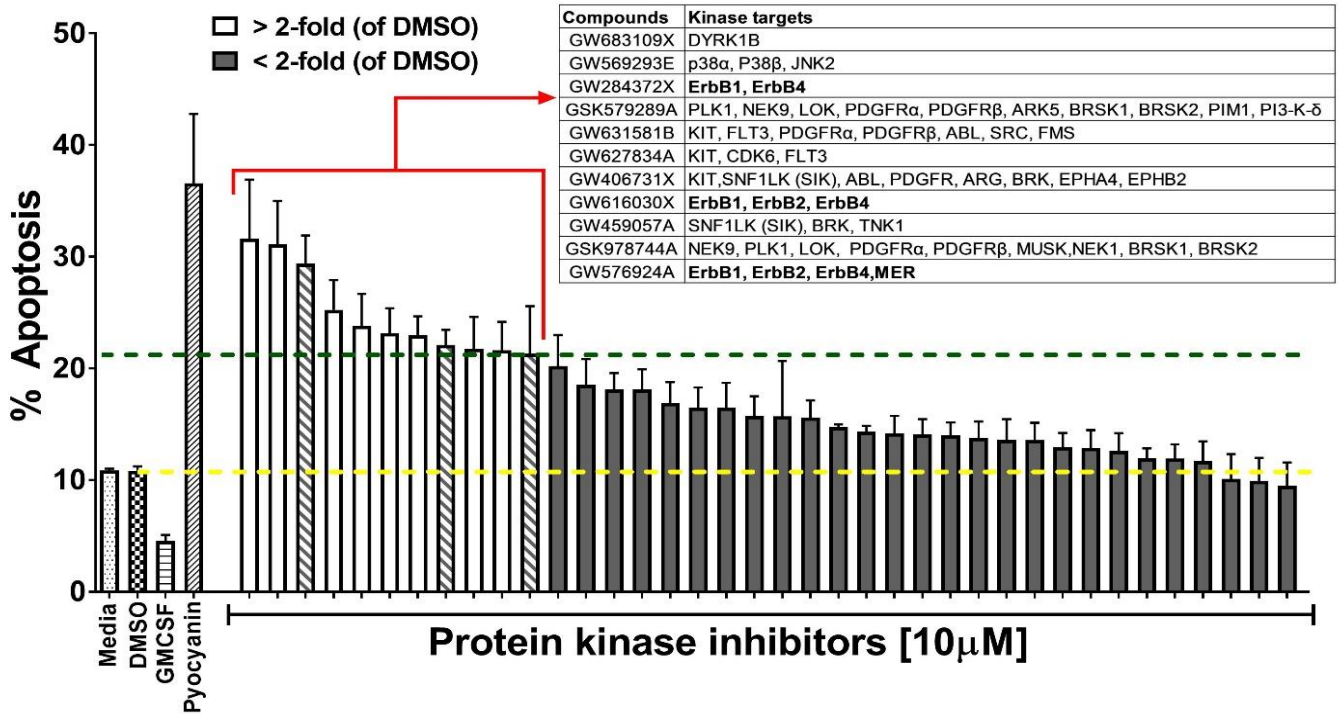


Figure 4.7. Validation of the primary screen. Ten compounds that had no acceleration of apoptosis in the primary screen round were re-screened for assessing the reliability and robustness of the primary screen. Neutrophils were incubated with these 10 kinase inhibitor compounds [10 μM], GMCSF [50 U/ml] and Pyocyanin [50 μM] for 6 hours followed by an assessment of apoptosis as discussed for primary screening. Among the compounds, only 1 [as denoted by the black bar] accelerated apoptosis ≥ 2-fold [the green dotted line] compared to DMSO (vehicle) control [the yellow dotted line]. Media is the negative control; DMSO is the vehicle control for inhibitor compounds, respectively. Data represent 3 independent experiments using 3 different donors with duplicate measurements under each condition, and expressed as mean percent of apoptosis ± SEM.



Compounds	Kinase targets
GW683109X	DYRK1B
GW569293E	p38α, P38β, JNK2
GW284372X	**ErbB1, ErbB4**
GSK579289A	PLK1, NEK9, LOK, PDGFRα, PDGFRβ, ARK5, BRSK1, BRSK2, PIM1, PI3-K-δ
GW631581B	KIT, FLT3, PDGFRα, PDGFRβ, ABL, SRC, FMS
GW627834A	KIT, CDK6, FLT3
GW406731X	KIT,SNF1LK (SIK), ABL, PDGFR, ARG, BRK, EPHA4, EPHB2
GW616030X	**ErbB1, ErbB2, ErbB4**
GW459057A	SNF1LK (SIK), BRK, TNK1
GSK978744A	NEK9, PLK1, LOK, PDGFRα, PDGFRβ, MUSK,NEK1, BRSK1, BRSK2
GW576924A	**ErbB1, ErbB2, ErbB4,MER**

Figure 4.8. Secondary screening of kinase inhibitor compounds on neutrophil apoptosis. Of the 62 compounds identified in the primary screening, 38 compounds that have relatively higher specificity for their kinase targets were tested in the same apoptosis assay used for the primary screen using 3 independent donors. Neutrophils were incubated with these inhibitor compounds [10 μM], GMCSF [50 U/ml] and Pyocyanin [50 μM] for 6 hours followed by an assessment of apoptosis by flow cytometry as discussed for the primary screen. Secondary screen yielded 11 compounds [as denoted by the white bars] that robustly accelerated apoptosis ⩾2 fold [the green dotted line] compared to DMSO (vehicle) control [the yellow dotted line]. The hatched bars represent the inhibitors that are highly selective for ErbB kinases. The kinase targets of those 11 compounds are shown in the inset table. Data represent 3 independent experiments using 3 different donors with duplicate measurements under each condition, and expressed as mean percent of apoptosis ± SEM. Media and DMSO represent the negative control and vehicle control for inhibitor compounds.

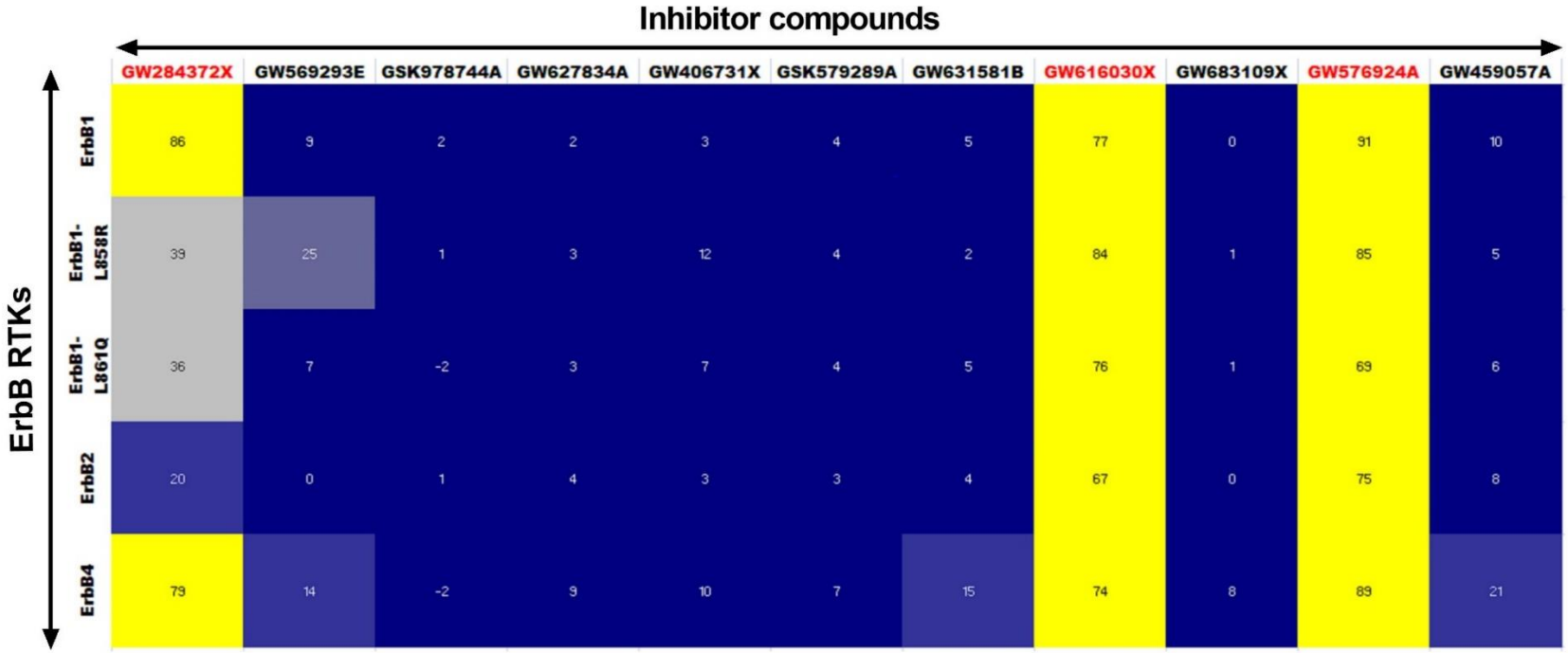


Figure 4.9. Specificity of the 11 hit compounds for ErbB RTKs. From the PKIS specificity profile (Elkins et al., 2016), the selectivity of the 11 hit compounds (from the secondary screen) for ErbB RTKs are shown in the hit map. The gradient of the yellow to blue colour represents high inhibitory (100%) to no (0%) inhibitory activities. Out of the 11 inhibitor compounds, 3 (red coloured) are highly specific for ErbB RTKs (ErbB1/2/4).

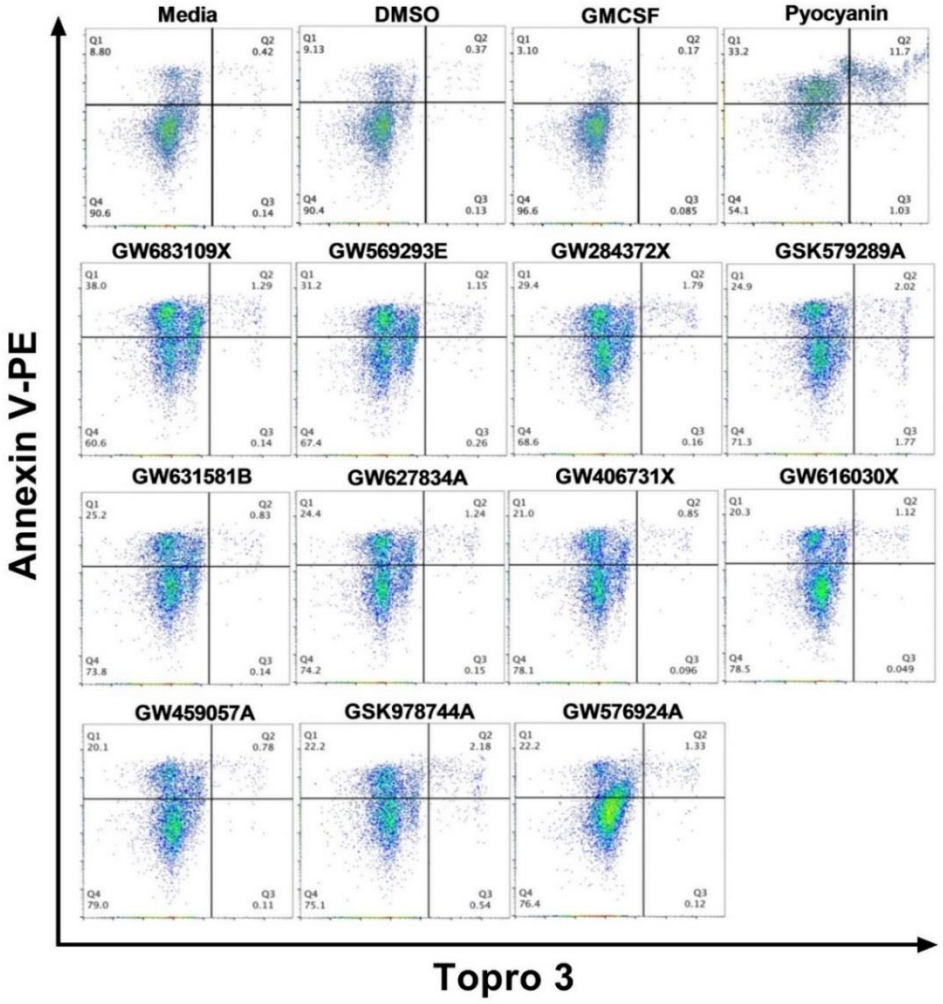


Figure 4.10. Secondary screening of kinase inhibitor compounds on neutrophil apoptosis. Of the 62 compounds identified in the primary screening, 38 compounds that have relatively higher specificity for their kinase targets were tested in the same apoptosis assay used for the primary screen using 3 independent donors. Neutrophils were cultured with these inhibitors [10 μM], GMCSF [50 U/ml] and Pyocyanin [50 μM] for 6 hours followed by staining with annexin V-PE/Topro-3 and neutrophil apoptosis assessed by flow cytometry. Quadrant plots from a representative experiment show the distribution of annexin V and Topro-3 in neutrophils for controls (media, DMSO, GMCSF and Pyocyanin) and the 11 compounds that accelerated ≥ 2-fold apoptosis over DMSO control. Media is the negative control; DMSO is the vehicle control for inhibitors. Apoptosis was determined by the summation of annexin-V single positive and annexin V/Topro-3 double positive events.

4.3. Discussion

Neutrophils are considered as the first cells to rapidly migrate to the site of infection (or inflammation) to eradicate infection or foreign particles, and soon after performing their function they undergo apoptosis followed by macrophage efferocytosis (El Kebir and Filep, 2013b, Savill et al., 1989b, Whyte et al., 1999). This controlled inflammation resolution process is maintained in normal physiology, but yet this process is dysregulated in inflammatory diseases such as COPD (Hoenderdos and Condliffe, 2013, Zhang et al., 2012). Current therapies do not effectively prevent or reverse neutrophil dominant inflammation and lung destruction, instead agents such as bronchodilators or glucocorticoids may reduce symptoms or exacerbation frequency (Rennard, 2004, Sutherland and Cherniack, 2004). Although there have been advances in the field of neutrophil apoptosis mechanisms, understanding aberrant apoptosis responses in COPD neutrophils and identifying novel molecular targets regulating apoptosis are warranted in designing effective treatment strategies for chronic inflammatory disease.

Systemic inflammation is observed in COPD patients with severe disease and during exacerbations, as evidenced by changes in circulating cytokines and chemokines as well as by phenotypic changes in circulatory cells (Agusti et al., 2003, Burnett et al., 1987, Gan et al., 2004, Milara et al., 2012, Sapey et al., 2011, Woolhouse et al., 2005, Wouters et al., 2007). This implies that the inflammatory response is not restricted to the lung environment. Furthermore, systemic inflammation is shown to correlate with the degree of deterioration of lung function (Donaldson et al., 2005, Hurst et al., 2006). Circulatory neutrophils from both stable COPD patients (Koenderman et al., 2000) and during COPD exacerbations (Oudijk et al., 2006, Oudijk et al., 2005) have been shown to be more responsive to neutrophil priming factors, leading to enhanced degranulation and oxidative burst activity. In a rat model, it has been shown that primed neutrophils more readily migrate to the pulmonary vasculature (Yoshida et al., 2006). These observational data and evidence showing prolonged survival responses of circulatory

neutrophils both in stable COPD patients (Zhang et al., 2012) and during exacerbations (Pletz et al., 2004, Schmidt-Ioanas et al., 2006) implie that circulatory neutrophils in COPD settings may develop altered phenotypes. To investigate a defect in the neutrophil apoptosis pathway in COPD circulatory neutrophils, I compared the rates of apoptosis between neutrophils from COPD versus healthy control subjects and found that while COPD neutrophils were more resistant to pyocyanin induced neutrophil apoptosis at 6 hours, there were no differences in any other treatment condition, including rates of spontaneous apoptosis. This difference in neutrophil apoptosis following pyocyanin treatment was possibly because of reduced ROS generation, as pyocyanin has been noted to induce neutrophil apoptosis via generation of ROS (Usher et al., 2002). This is not supported by the literature since COPD neutrophils have been shown to undergo enhanced oxidative burst activity (Noguera et al., 2001).

Pyocyanin has been noted to induce neutrophil apoptosis *in vitro* and *in vivo* (Allen et al., 2005a, Usher et al., 2002). It has been further shown that pyocyanin-induced neutrophil apoptosis impairs neutrophil-mediated host defence (Allen et al., 2005a). Therefore, following pyocyanin treatment the differential apoptosis in COPD neutrophils observed in this thesis may show different outcomes of infection in the lung in the disease settings.

One of a major limitation in the comparison of neutrophil apoptosis in COPD patients and healthy control subjects is that, as discussed in chapter 3, the blood samples for both groups of the study subjects were travelled from Manchester to Sheffield, which may cause an pre-activation of neutrophils and thefore may have an impact on the rates of apoptosis observed.

Although GMCSF is a known neutrophil survival stimulus (Yasui et al., 2002), no prosurvival effects were observed in neutrophils from COPD patients and healthy control subjects studied in this chapter (Figure 4.1). This could perhaps be explained by possible pre-activation of neutrophils because of blood travelling from Manchester (as discussed above and in chapter 3). However, I found neutrophil survival in response to GMCSF in

COPD and healthy subjects, which were recruited from Sheffield (see Chapter 5: Figure 5.17, 5.18 and 5.19).

The human kinome is considered to be a rich resource of candidates of pharmacologically tractable proteins that play vital roles in most cellular processes. Kinases are implicated in a broad range of disease phenotypes (Lahiry et al., 2010) and kinase inhibitors are being increasingly considered as therapeutics in clinical use (Wu et al., 2015). An extensive body of literature suggests that previously therapeutically unreported kinases may provide clues in designing new and effective therapeutics in treating multiple diseases, including cancer and inflammatory diseases (Fedorov et al., 2010). Kinases are implicated in neutrophil apoptosis (Burgon et al., 2014, Juss et al., 2012, Rossi et al., 2006, Wang et al., 2003, Webb et al., 2000) and promoting neutrophil apoptosis has been shown to enhance inflammation resolution (Burgon et al., 2014, Chello et al., 2007, Heasman et al., 2003, Ren et al., 2008, Rossi et al., 2006). The neutrophil kinome has not been clearly defined at a functional level and, to this end, I have screened a PKIS compound library in models of neutrophil cell death to identify previously unreported kinases that regulate neutrophil apoptosis and that may be therapeutically targetable for inflammatory diseases (Athens et al., 1961).

High-throughput screening (HTS) assays are invaluable in the screening of a large number of small molecules for assessing their activity in a diverse discipline in biological sciences (Inglese et al., 2007). High-throughput kinase inhibitor screens can be performed with ease to determine the potency and selectivity, and thus has proven beneficial for drug development across a multiple disease phenotypes (Goldstein et al., 2008, Grant, 2009). Here I employed a moderate/high-throughput assay for screening PKIS compounds based on a flow cytometer assisted neutrophil apoptosis assay as measured by the detection of phosphatidyl serine exposure (van Engeland et al., 1998). This flow cytometry based apoptosis detection process is a reliable apoptosis detection method and used routinely by researchers (van Engeland et al., 1998). The primary screening resulted in 62 compounds that accelerated apoptosis ≥ 2-fold over control,

and out of which 38 compounds with the greatest selectivity were taken forward to the secondary screen round. This resulted in the identification of 11 inhibitor compounds that robustly accelerated neutrophil apoptosis.

In the primary screen, I performed the screen once for each inhibitor because of limiting the availability of the inhibitors; therefore, the primary screen might lead to false positive or false negative results. Moreover, the primary screen was performed at a concentration of 62 µM, which is far higher than the typical dose of drugs used in clinical medicine and therefore may result in off-target effects. Furthermore, the primary screen was executed accross 5 individual experiments performed on different days using 5 individual donors and thus the screen result might have been compromised by biological variability. As discussed above, because of the possibilities of obtaining false-negative data and biological variability in the primary screen, the screen process, therefore, might have missed some of the potential inhibitor hits. These are potential major limitations in the screening procedure employed.

Both the primary and the secondary screen were performed at a single timepoint of 6 hours, as the goal in this study was to identify PKIS compounds that accelerate apoptosis and constitutive death would be significantly increased at later timepoints, making this objective difficult. For the secondary screen round, the concentration of the inhibitors was reduced to 10 µM and the screen was repeated 3 times with the selected 38 inhibitors. The 38 compounds (among the 62 compounds from the primary screen result) with highest selectivity for their kinase targets were chosen based on previously published kinase selectivity profiles for the PKIS (Elkins et al., 2016). In addition to these, there were a number of very broad spectrum compounds in the library and although some of these were able to accelerate neutrophil apoptosis in the primary screen, it would have been very difficult to know which kinases were responsible. For this reason, broadly-specific compounds were excluded from the secondary screen. The secondary screen identified 11 inhibitor compounds that enhanced neutrophils apoptosis ≥ 2-fold and their kinase targets were identified. As these inhibitor compounds are selective for

one or very few kinase targets, these kinases can be considered as having a potential role in neutrophil apoptosis/survival pathway.

As described earlier, the primary screen was validated by including 10 compounds (that did not enhance apoptosis in the primary screen) and indeed, only one of these compounds did enhance neutrophil apoptosis in the secondary screen round, which was possibly lost as a hit in the primary screen because of a false negative effect. The primary and secondary screening rounds identified kinases with well-described roles in regulating neutrophil apoptosis, which gave us confidence in the design and robustness of the screen. These included p38, PI3K, and CDK6. The role of p38 and PI3K/AKT in neutrophil apoptosis/survival axis regulation is well described (Alvarado-Kristensson et al., 2004, Derouet et al., 2004, Klein et al., 2000, Moulding et al., 1998). CDK inhibitors have been reported to induce neutrophil apoptosis *in vitro* and in multiple *in vivo* models in an Mcl-1 dependent mechanism (Dorward et al., 2017, Rossi et al., 2006). Furthermore, CDKs inhibition has also been noted to alter apoptosis of terminally differentiated neuronal cells (Monaco and Vallano, 2003).

Further to these kinases with known roles in neutrophil apoptosis/survival pathways, a number of kinases identified in the screen have been reported to play roles in cell survival phenotypes in alternative cellular contexts, and therefore may also have potential roles in regulating neutrophil apoptosis/survival pathways. For example, PDGFR was the most frequent target of the identified compounds, although the targeted compounds were relatively less specific. A number of studies have suggested that PDGFR signalling is an essential component in apoptosis pathways in diverse cellular contexts (Cenciarelli et al., 2014, Rizvi et al., 2014, Romashkova and Makarov, 1999). Another important kinase target identified was KIT that has also been reported to play an important role in survival of mast cells (Iemura et al., 1994, Juurikivi et al., 2005) and leukaemic cells (Belloc et al., 2009). DYRK1B was also another important kinase that has been reported to play roles in cell survival in a number of carcinomas (Deng et al., 2006, Gao et al., 2009, Jin et al., 2005, Mercer et al., 2006). These and other kinases

identified in the screen (inset table Figure 4.8) may have potential to regulate neutrophil survival pathway, however, further studies are required for the validation of their role in neutrophil cell death pathways and in inflammation.

The ErbB family of RTKs were the second most frequent targets and crucially, were targeted by the most selective compounds, including the compound that was the third most effective at driving apoptosis in the secondary screen (GW284372X). ErbB is part of a family of four structurally and closely related RTKs that have been implicated in the regulation of multiple cell functions, including cell proliferation, survival, apoptosis etc., and ErbB family kinases are exploited as pharmacologic targets in cancer therapy (Roskoski, 2014). Since the ErbB family of RTKs were the frequent targets of highly specific inhibitor compounds, it is more likely that this family of RTKs have roles in neutrophils survival. Since other kinases targeted by a frequent number of inhibitors (e. g. PDGFRs, KIT) were inhibited by broad-spectrum inhibitor compounds, and therefore, it is very difficult to know which kinase is responsible for pro-survival response. Thus, these kinases were deprioritised for further studies to exploit their roles in neutrophil survival. In chapter 5 in this thesis, I will go on to investigate the roles of ErbB RTKs in neutrophil survival and inflammation.

To confirm the activation of ErbB family kinases in response to survival signals, I also interrogated the activation of ErbB family kinases in response to N^6-MB-cAMP (a cAMP analog and known to enhance neutrophil survival) from a kinase antibody microarray profiling by our research group (**Appendix 7.7**). The ErbB family of RTKs were found to be activated in response to N^6-MB-cAMP (**Appendix 7.7**), suggesting that ErbB members are potential regulators of neutrophils survival.

Since enhancing neutrophil apoptosis contribute to inflammation resolution (Burgon et al., 2014, Chello et al., 2007, Heasman et al., 2003, Ren et al., 2008, Rossi et al., 2006), this strategy can be beneficial for treating inflammatory diseases such as COPD. It is well-established that neutrophil apoptosis in the lung of COPD is delayed because of the

presence of pro-survival factors in the lung microenvironment and this may contribute to the pathogenicity of COPD (Brown et al., 2009, Haslett, 1999, Zhang et al., 2012). Therefore, the inhibition of the potential kinases identified from the screen can be exploited for developing therapies for COPD and other inflammatory diseases. As discussed earlier, circulatory neutrophil apoptosis both in stable COPD patients (Zhang et al., 2012) and during exacerbations (Pletz et al., 2004, Schmidt-Ioanas et al., 2006) were reported to be reduced. However, my study (in this chapter and in chapter 5) and another study (Noguera et al., 2004) showed no change in constitutive neutrophil apoptosis. As discussed in chapter 1, the study by Noguera et al and my current study could be limited by considerably lower number of patients studied than that of Pelz et al, Zhang et al and Schmidt-Ioanas et al. While enhancing neutrophil apoptosis could be therapeutically beneficial by reducing neutrophil burden in the lung of COPD patients, however, it has to be considered that the patients may be immune-compromised by reducing the circulating neutrophils.

As discussed, the kinase targets of the compounds were identified from the previously published activity profile data for PKIS (Elkins et al., 2016). Since the human kinome consists of 518 kinases (Manning et al., 2002) and activity profile data for PKIS was generated for only 224 human kinases that consist of previously targeted kinases and their close family members (Elkins et al., 2016), the profile represents only 40% of human kinome (224 out of 518). Therefore, the kinases used in the profile data under represent the whole human kinome. Thus, all the potential kinase targets of the identified compounds were not possible to identify in this study. This is one of the limitations of this study.

In conclusion, my approach combing the kinase inhibitor screen data with the previously published kinase profile data resulted in the identification of some lead kinases that may play critical roles in neutrophil apoptosis/survival pathways. Further studies are warranted to disclose the roles of these potential kinases in the regulation of neutrophil apoptosis/survival pathways and inflammation. Thus, this study opens up the

possibility to identify kinases associated with neutrophil apoptosis pathways that therefore may serve as targets for novel therapeutics for inflammatory diseases.

Chapter 5: ErbB family kinases are important regulators of neutrophil survival and inflammation.

5.1. Brief Introduction

As described in Chapter 4, a kinase inhibitor library screen identified the ErbB family of RTKs as potential regulators of neutrophil survival pathways. The ErbB family consists of four structurally and functionally related RTKs including ErbB1 (EGFR), ErbB2 (Her 2, Neu), ErbB3 (Her 3) and ErbB4 (Her 4), and this family have been implicated in the regulation of cell survival and proliferation (Casalini et al., 2004). As oncoproteins they are involved in the development of various human cancers (Roskoski, 2014). Small molecule inhibitors and monoclonal antibodies (mAb) to the ErbB family kinases are used in clinical practice in cancer medicine (Singh et al., 2016). Apart from their role in the development of cancer, the ErbB RTKs are also reported to play roles in inflammatory diseases in the lung, gut, and skin (Davies et al., 1999, Finigan et al., 2011, Frey and Brent Polk, 2014, Hamilton et al., 2003, Pastore et al., 2008). In a bleomycin-induced acute lung injury model, ErbB2 was reported to be upregulated in whole lung lysates, and also ErbB1 ligands were noted to be enhanced in BAL sample collected from ALI (Acute Lung Injury) patients (Finigan et al., 2011). In addition, ErbB3 expression was also shown to be upregulated in bronchial biopsies in long-term smokers compared to non-smokers (O'Donnell et al., 2004). These observational studies in the context of lung inflammation suggest that ErbB signalling may play an important role in lung inflammation. There is no literature reporting the role of the ErbB kinases in human neutrophils, except for one publication which shows that circulatory neutrophils express ErbB1 in response to inflammatory stimuli (Lewkowicz et al., 2005). I hypothesise that ErbB RTKs are important regulators of neutrophil survival and inflammation, and may be considered as potential therapeutic targets for inflammatory diseases. These hypotheses were addressed by testing the effect of ErbB inhibitors on human neutrophil apoptosis *in vitro* and on neutrophilic inflammation *in vivo.*

5.2. Results

5.2.1. ErbB inhibitors Erbstatin analog and Tyrphostin AG825 induced neutrophil apoptosis.

To investigate the role of the ErbB family of RTKs in regulating neutrophil apoptosis *in vitro*, I used two commercially available small molecule inhibitors Erbstatin analog and Tyrphostin AG825, which are selective, ATP-competitive inhibitors for ErbB1 and ErbB2, respectively (Osherov et al., 1993, Umezawa and Imoto, 1991). The Erbstatin analog inhibits ErbB1>2, while the Tyrphostin AG825 inhibits ErbB2>1 (Osherov et al., 1993, Umezawa and Imoto, 1991). These compounds were incubated at a range of concentrations with freshly isolated neutrophils from healthy subjects *in vitro,* and their effects on neutrophil apoptosis were measured by light microscopy. Erbstatin analog significantly accelerated neutrophil apoptosis as assessed by light microscopy in a dose-dependent manner at 40 µM at 6 hours [p=0.002, % Apoptosis changed from 27.00±4.63 (DMSO) to 61.43±5.68 (40 µM Erbstatin analog)] (**Figure 5.1**). In the same way, Tyrphostin AG825 also significantly enhanced neutrophil apoptosis at 6 hours at 50 and 100 µM [p=0.0003 and 0.0001 respectively, % apoptosis changed from 15.26±1.87 (DMSO) to 41.03±4.6 (50 µM Tyrphostin AG825) and 53.02±9.77 (100 µM Tyrphostin AG825)] (**Figure 5.2: A**), and significance was also reached at 50 µM at 20 hours [p=0.0001, % Apoptosis changed from 61.88±3.76 (DMSO) to 95.72±1.10 (Tyrphostin AG825)] (**Figure 5.2: B**). The effects of these two inhibitors on neutrophil apoptosis were validated biochemically by measuring PS exposure by Annexin-V and Topro-3 (Vermes et al., 1995), where both Erbstatin analog and Tyrphostin AG825 significantly increased the number of Annexin-V positive cells at 6 hours (p=0.0002 and p=0.0091, respectively) (**Figure 5.3 and 5.4**). It is noteworthy to mention that the cell death observed with Erbstatin analog or Tyrphostin AG825 treatment was not because of necrotic cell death, as Topro-3 positivity was not significantly changed. To determine whether Erbstatin analog- and Tyrphostin AG825-induced neutrophil apoptosis is caspase-dependent, the inhibitors were incubated in the presence or absence of the pan-caspase inhibitor, Q-VD-OPh hydrate (QVD) (Wardle et al., 2011). QVD completely abrogated Erbstatin

analog- and Tyrphostin AG825-induced neutrophil apoptosis at both time-points ($p<0.01$ for Erbstatin analog; $p<0.01$ and $p<0.001$ for Tyrphostin AG825) (**Figure 5.5 and Figure 5.6**).

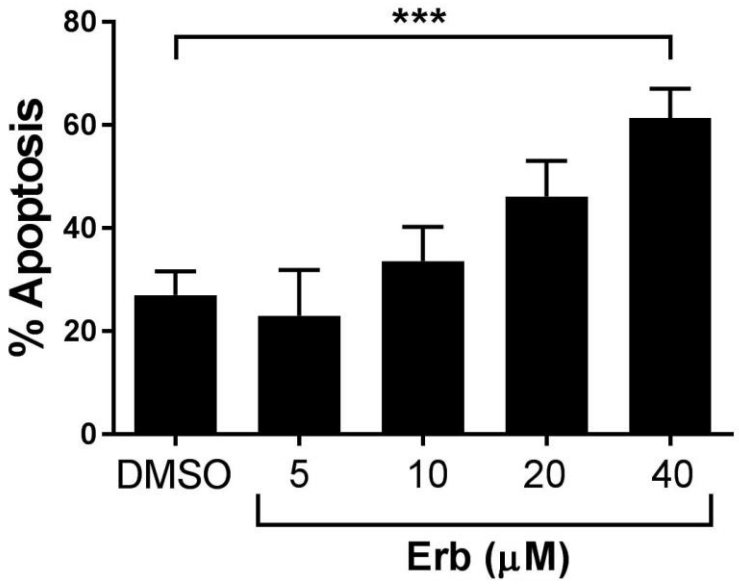


Figure 5.1. Erbstatin analog dose dependently increased neutrophil apoptosis. Neutrophils from healthy subjects were treated with or without Erbstatin analog (Erb) at a range of concentrations [0, 5, 10, 20, 40 μM] for 6 hours followed by an assessment of apoptosis by light microscopy. Data represent a combination of four independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. DMSO is the vehicle control for Erbstatin analog. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Dunnett's post-hoc test, and presented as ***$p<0.001$.

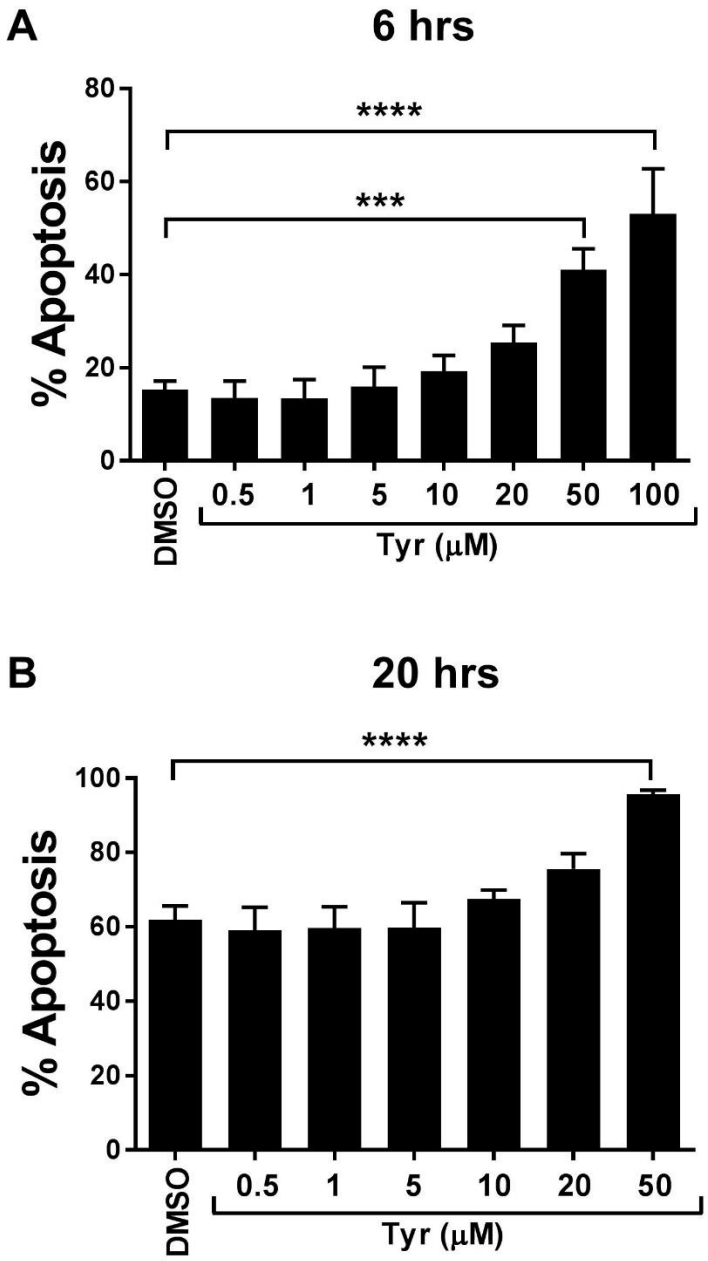


Figure 5.2. Tyrphostin AG825 dose dependently increased neutrophil apoptosis. Neutrophils from healthy subjects were treated with or without Tyrphostin AG825 (Tyr) at a range of concentrations [0, 0.5, 1, 5, 10, 20, 50, 100 μM] for 6 (A) or 20 (B) hours followed by an assessment of neutrophil apoptosis by light microscopy. Data represent a combination of 6 (A) or 5 (B) independent experiments with duplicate measurements under each condition, and the values are expressed as mean percentage apoptosis ± SEM. DMSO is the vehicle control for Tyrphostin AG825. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Dunnett's post-hoc test, and presented as ***p<0.001, ****P<0.0001.

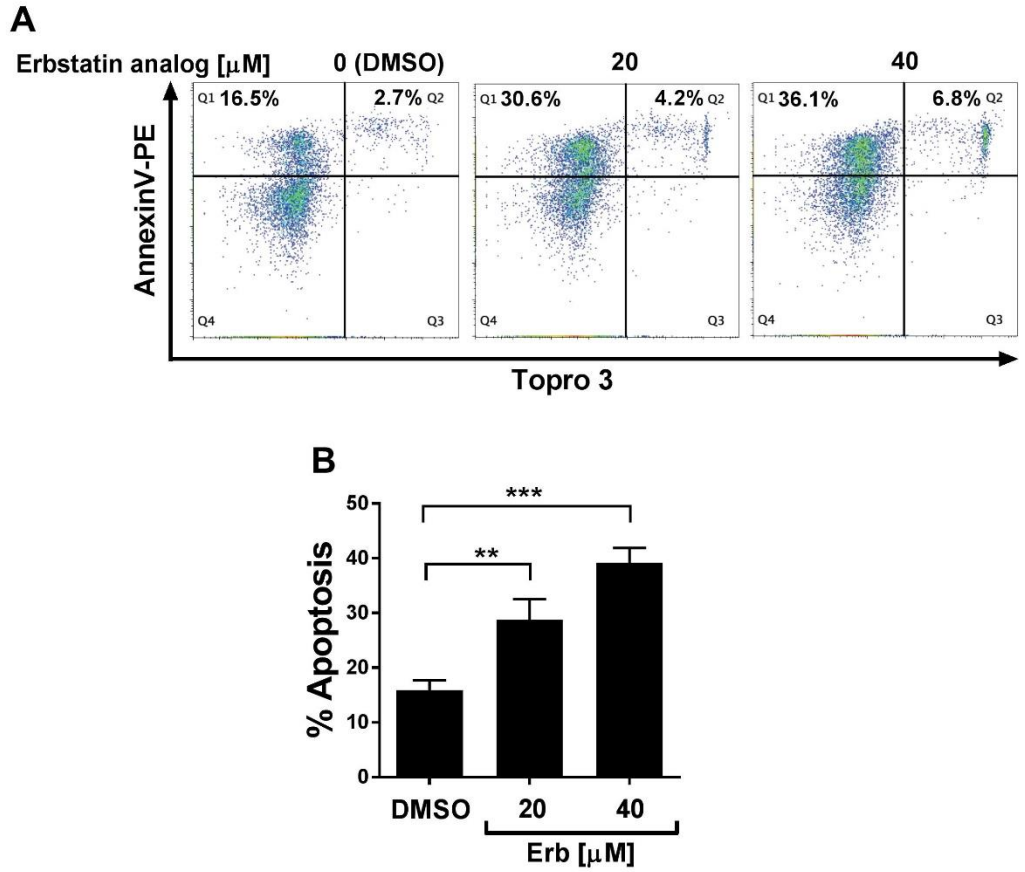


Figure 5.3. Erbstatin analog induced neutrophil apoptosis was validated by flow cytometry. Neutrophils from healthy subjects were treated with or without Erbstatin analog [Erb, 0, 20, 40 μM] for 6 hours followed by staining neutrophils with annexin V-PE and Topro-3 for the assessment of apoptosis by flow cytometry. (A) Quadrant plots of a representative experiment showing the effects of Erbstatin analog [Erb, 0, 20, 40 μM] on the distribution of Annexin V-PE and Topro-3 in neutrophils. Apoptosis was assessed by the summation of the annexin-V single positive and the annexin V/Topro-3 double positive events. (B) A graph showing the effects of Erbstatin analog on neutrophil apoptosis are presented. Data represent 6 independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. DMSO is the vehicle control for Erbstatin analog. Statistical significances were calculated by one way ANOVA followed by Dunnett's post-hoc test and presented as **p<0.01, ***p<0.001.

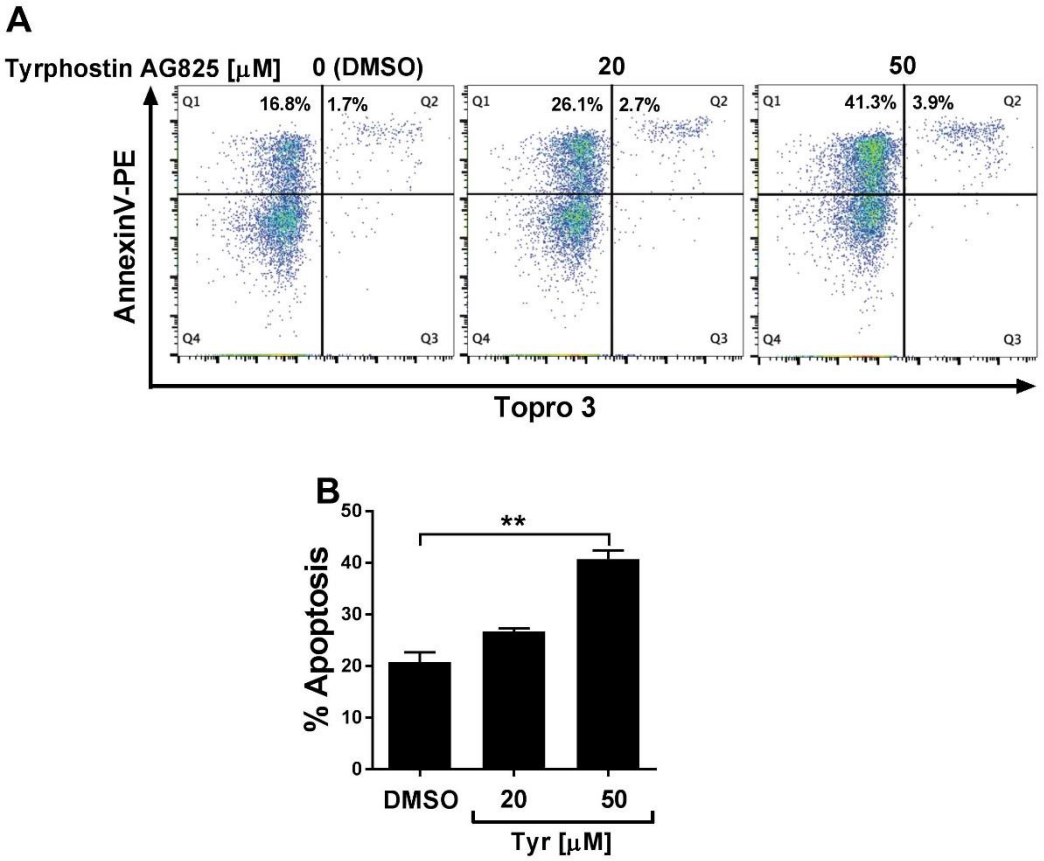


Figure 5.4. Tyrphostin AG825-induced neutrophil apoptosis was validated by flow cytometry. Neutrophils from healthy subjects were treated with or without Tyrphostin AG825 [Tyr, 0, 20, 50 μM] for 6 hours followed by staining neutrophils with annexin V-PE and Topro-3 for the assessment of apoptosis by flow cytometry. (A) Quadrant plots of a representative experiment showing the effects of Tyrphostin AG825 [Tyr, 0, 20, 50 μM] on the distribution of Annexin V-PE and Topro-3 in neutrophils. Apoptosis was assessed by the summation of the annexin-V single positive and the annexin V/Topro-3 double positive events. (B) A graph showing the effects of Tyrphostin AG825 on neutrophil apoptosis are presented. Data represent 3 independent experiments with duplicate measurements under each condition, and the values presented in each condition are presented as mean percent apoptosis ± SEM. DMSO is the vehicle control for Tyrphostin AG825. Statistical significances were calculated by one way ANOVA followed by Dunnett's post-hoc test and presented as **$p<0.01$.

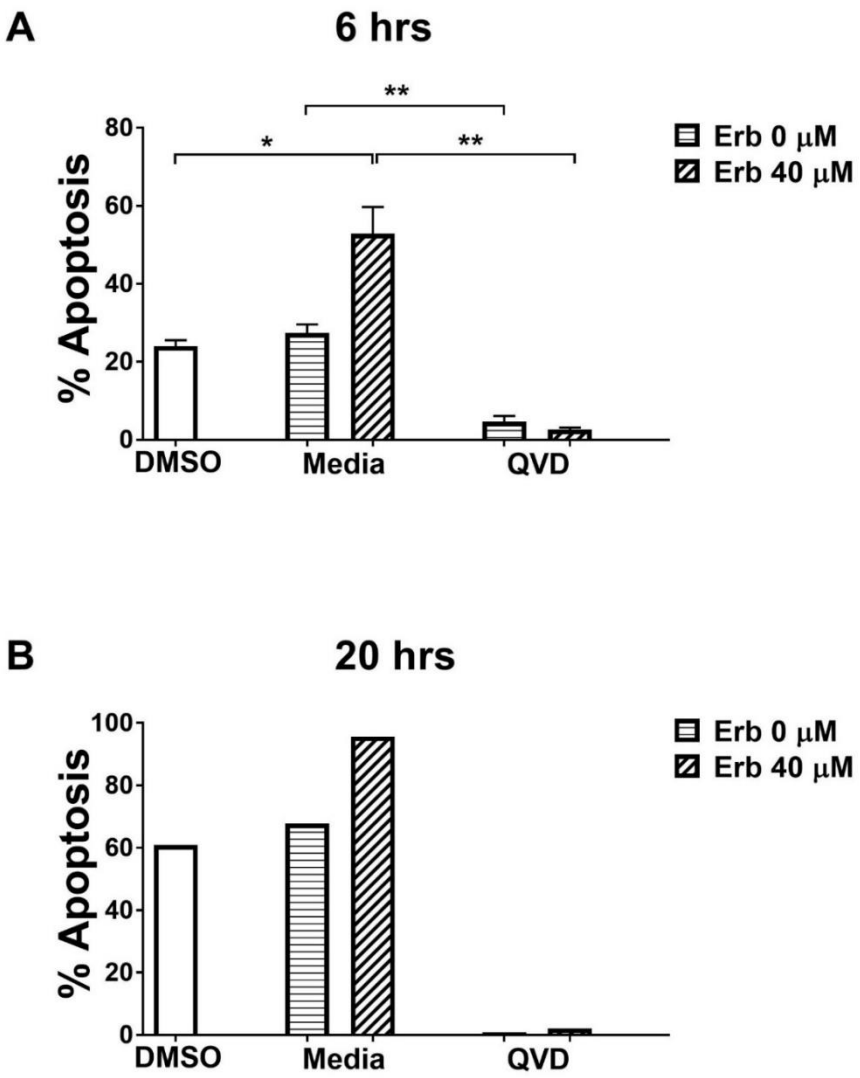


Figure 5.5. Erbstatin analog-induced neutrophil apoptosis is caspase-dependent. Neutrophils from healthy subjects were treated with or without Erbstatin analog [Erb, 0, 40 μM] in the presence or absence of pan-caspase inhibitor, Q-VD-OPh [QVD, 1 μM] for 6 (A) or 20 (B) hours followed by an assessment of apoptosis by light microscopy. Data represent 5 (A) or 1 (B) independent experiment(s) with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. Media is the negative control; DMSO is the vehicle control for Erbstatin analog. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *$p<0.05$, **$p<0.01$.

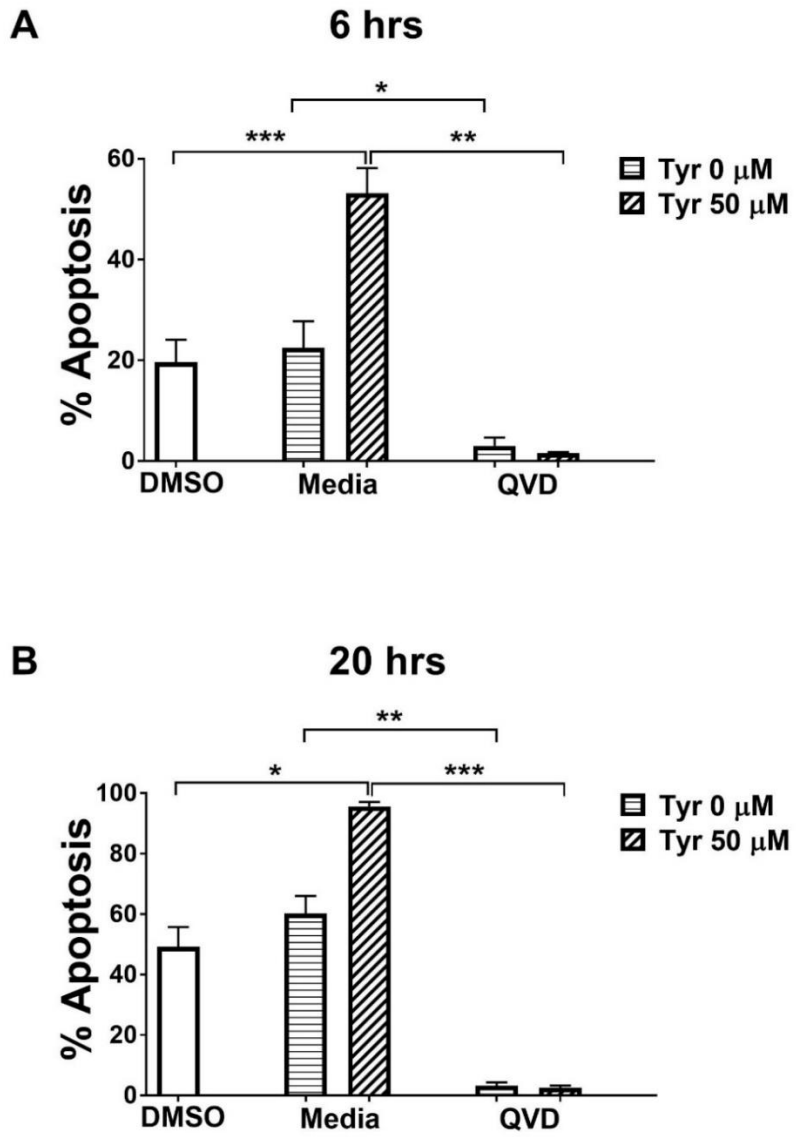


Figure 5.6. Tyrphostin AG825 induced neutrophil apoptosis is caspase-dependent. Neutrophils from healthy subjects were treated with or without Tyrphostin AG825 [Tyr, 0, 50 μM] in the presence or absence of pan-caspase inhibitor, Q-VD-OPh [QVD, 1 μM] for 6 (A) or 20 (B) hours followed by an assessment of apoptosis by light microscopy. Data represent 4 independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. Media is the negative control; DMSO is the vehicle control for Tyrphostin AG825. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Neutrophilic inflammation is a prominent feature of COPD partially because of prolonged neutrophil lifespan within the inflammatory lung microenvironment (Hoenderdos and Condliffe, 2013, Zhang et al., 2012). Moreover, a broad range of literature suggests circulatory neutrophils from COPD patients are functionally altered, including altered adhesion and migration in response to inflammatory stimuli (Burnett et al., 1987, Milara et al., 2012, Sapey et al., 2011, Woolhouse et al., 2005), reduced phagocytosis capacity (Fietta et al., 1988, Prieto et al., 2001), and increased ROS generation (Noguera et al., 2001). Therefore, neutrophils were isolated from COPD patients and age-matched healthy control subjects, and incubated with Erbstatin analog or Tyrphostin AG825 for 6 or 20 hours (Demographic characteristics of COPD patients and age-matched healthy control subjects are presented in **Table 5.1)**. Both Erbstatin analog and Tyrphostin AG825 dose-dependently enhanced neutrophil apoptosis in COPD and age-matched healthy subjects at 6 and 20 hours [Erbstatin analog: $p<0.0001$ (HC), $p=0.0043$ (COPD) for 6 hours and $p=0.0003$ (HC), $p<0.0001$ (COPD) for 20 hours; Tyrphostin AG825: $p=0.0003$ (HC), $p<0.0001$ (COPD) for 6 hours and $p=0.0002$ (HC), $p<0.0001$ (COPD) for 20 hours] (**Figure 5.7 and 5.8**).

Table 5.1: Demographic characteristics of COPD patients and age-matched healthy control subjects (Sheffield Cohort). Data are expressed as mean ± SEM or number. FEV1= Forced expiratory volume in the first second; FVC=Forced Vital Capacity.

Characteristics	COPD	Healthy subjects (HC)
Number of patients (n)	10	7
Age years (yrs)	61.70 ± 2.30	66.00 ± 3.56
Female/Male (n)	4/6	2/5
FEV1 (L)	1.68 ± 0.27	2.96 ± 0.27
FEV1 (%)	62.00 ±7.59	114.40 ± 7.91
FEV1/FVC (%)	57.00 ± 4.92	74.43 ± 1.70
Smoking Status (Current/Ex/Never)	3/7/0	1/0/6
Pack years	38.40 ± 5.70	0.57 ± 0.57
Exacerbations in last 1 year	3.80 ± 1.08	-

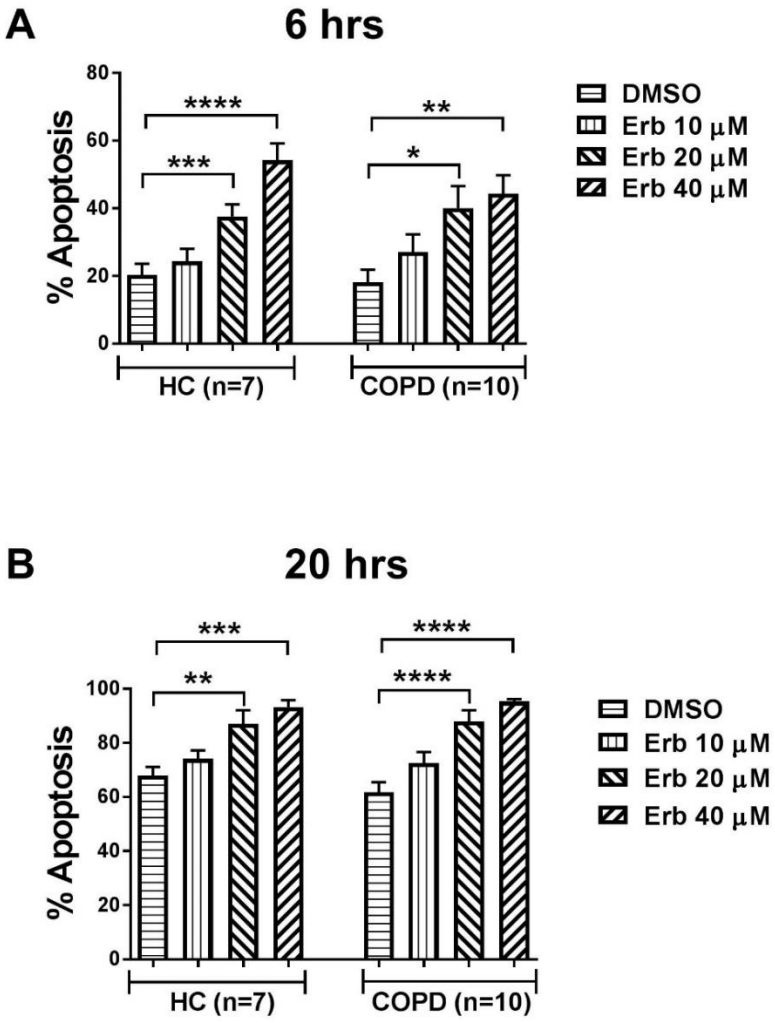


Figure 5.7. Erbstatin analog promoted neutrophil apoptosis in COPD patients and age-matched healthy subjects. Neutrophils from COPD patients and age-matched healthy (HC) subjects were cultured in the presence or absence of Erbstatin analog [Erb, 0, 10, 20 and 40 µM] for 6 (A) or 20 (B) hours followed by an assessment of apoptosis by light microscopy. Experiments were performed with duplicate measurements in each condition, and the values presented in each condition are expressed as mean percent apoptosis ± SEM, n=10 (COPD), 7 (HC). DMSO is the vehicle control for Erbstatin analog. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Dunnett's post-hoc test and presented as *p<0.05, **p<0.01, ***p<0.001, ****P<0.0001.

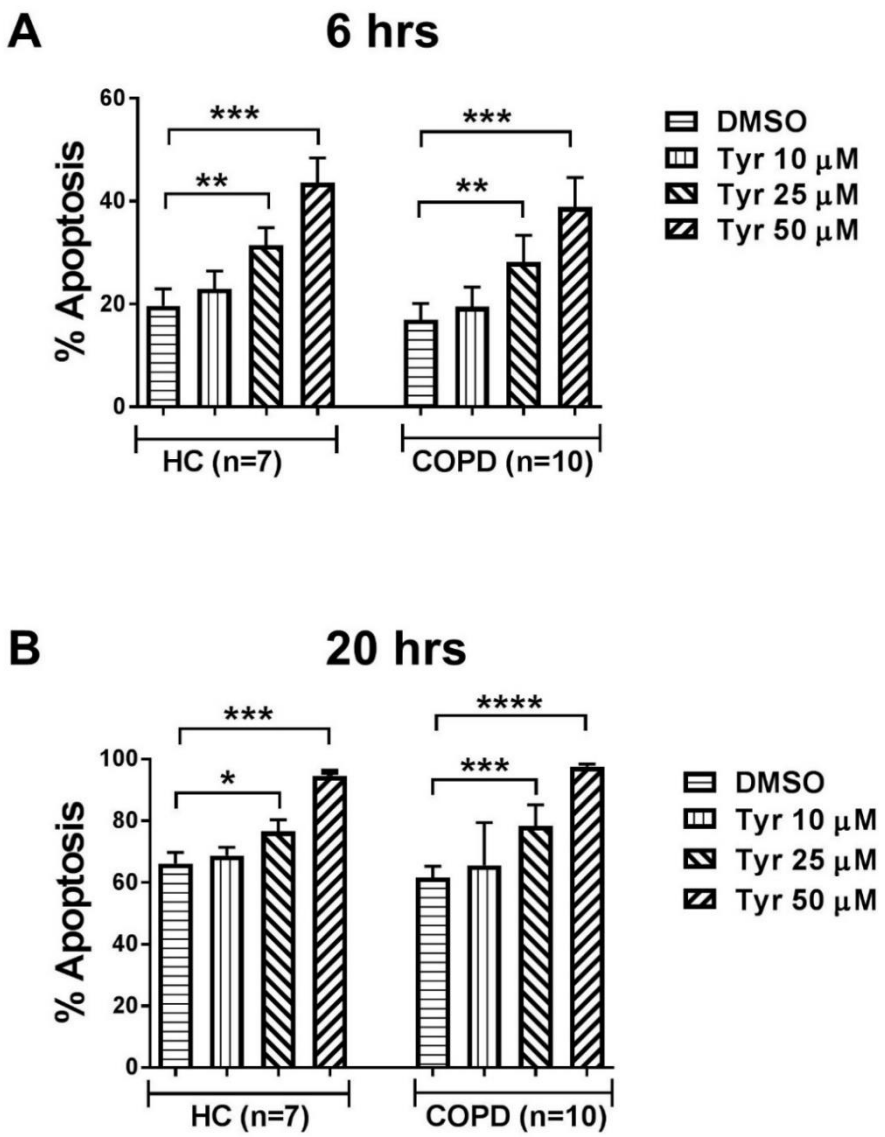


Figure 5.8. Tyrphostin AG825 promoted neutrophil apoptosis in COPD patients and age-matched healthy subjects. Neutrophils from COPD patients and age-matched healthy (HC) subjects were cultured in the presence or absence of Tyrphostin AG825 [Tyr, 0, 10, 25 and 50 µM] for 6 (A) or 20 (B) hours followed by an assessment of apoptosis by light microscopy. Experiments were performed with duplicate measurements in each condition, and the values presented in each condition are expressed as mean percent apoptosis ± SEM, n=10 (COPD), 7 (HC). DMSO is the vehicle control for Tyrphostin AG825. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Dunnett's post-hoc test and presented as *p<0.05, **p<0.01, ***p<0.001, ****P<0.0001.

5.2.2. Epidermal Growth Factor (EGF) and Neuregulin-1 (NRG-1) did not affect neutrophil apoptosis.

Ligand binding to the extracellular ligand binding site can lead to the activation of ErbB kinases (Roskoski, 2014). Therefore, apoptosis is expected to decrease in the presence of ligands, and to test this, neutrophils were co-cultured with or without known ligands such as EGF (a well-known ligand for ErbB1) and Neuregulin-1 (a known ligand for ErbB3 or ErbB4). However, EGF did not have any effect on neutrophil apoptosis when neutrophils treated with a range of EGF concentrations (1-1000 ng/ml) for 20 hours (**Figure 5.9 A**). Furthermore, Neuregulin-1 did not affect the apoptosis rates at 6 and 20 hours ($p=0.999$ and $p=0.715$) (**Figure 5.9 B&C**). ErbB kinases can form homodimers or heterodimers with other ErbB partners, and heterodimerisation with ErbB2 is the preferred dimerisation partner in ErbB signalling (Maruyama, 2014). Among the heterodimers of ErbB2, ErbB2/ErbB3 heterodimer generates the most potent mitogenic signals (Alimandi et al., 1995, Wallasch et al., 1995) and is linked with cell survival pathways (Kita et al., 1994, Soltoff et al., 1994). Therefore, I hypothesised that the ErbB2/ErbB3 heterodimer may participate in the neutrophil survival pathway. To test this, neutrophils were stimulated with or without an ErbB3 neutralization antibody followed by an assessment of apoptosis at 6 hours. Apoptosis was found to increase in the presence of the EbB3 neutralization antibody, however, it was not possible to do any statistical analysis as only two experiments were performed (**Figure 5.10**).

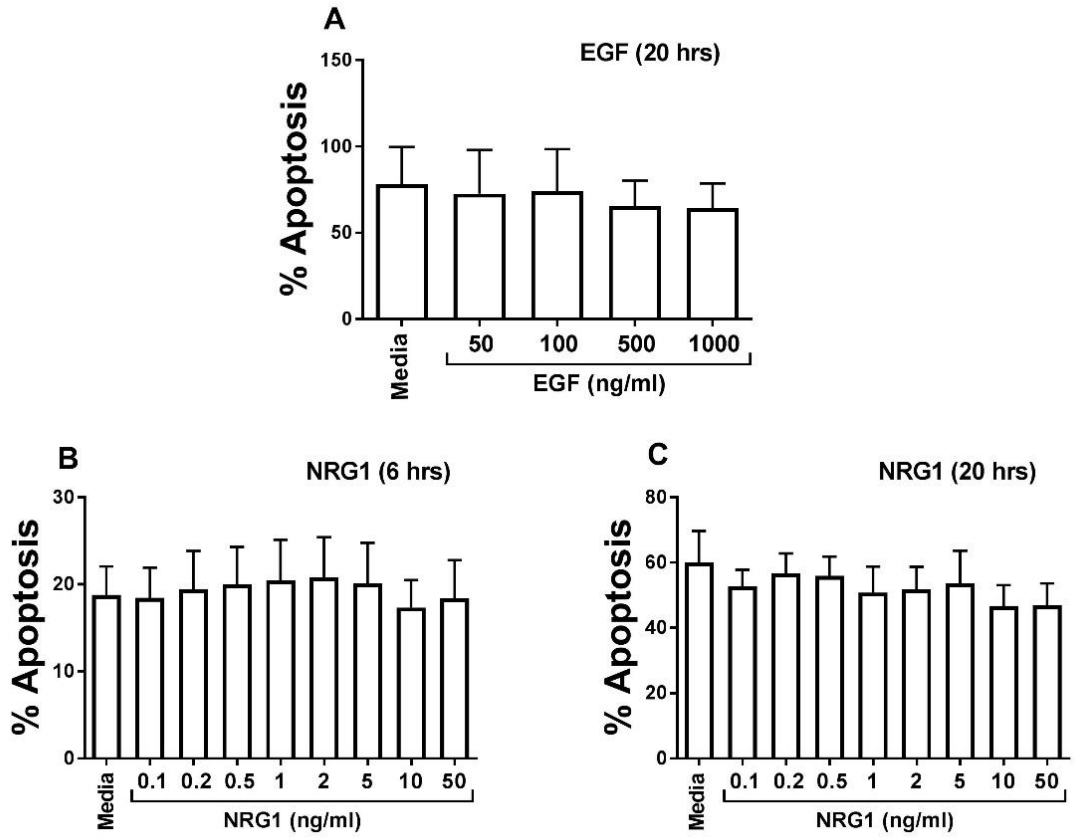


Figure 5.9. Stimulation of neutrophils with EGF (an ErbB1 ligand) or NRG1 (an ErbB3/4 ligand) did not affect apoptosis. Neutrophils from healthy subjects were treated with EGF [0, 50, 100, 500, 1000 ng/ml] for 20 hours (A) or NRG1 [0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 50.0 ng/ml] for 6 (B) and 20 (C) hours, followed by the assessment of apoptosis by light microscopy. Data represent 2 (A) or 3 (B, C) independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. Media is the negative control. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Dunnett's post-hoc test (for B, C).

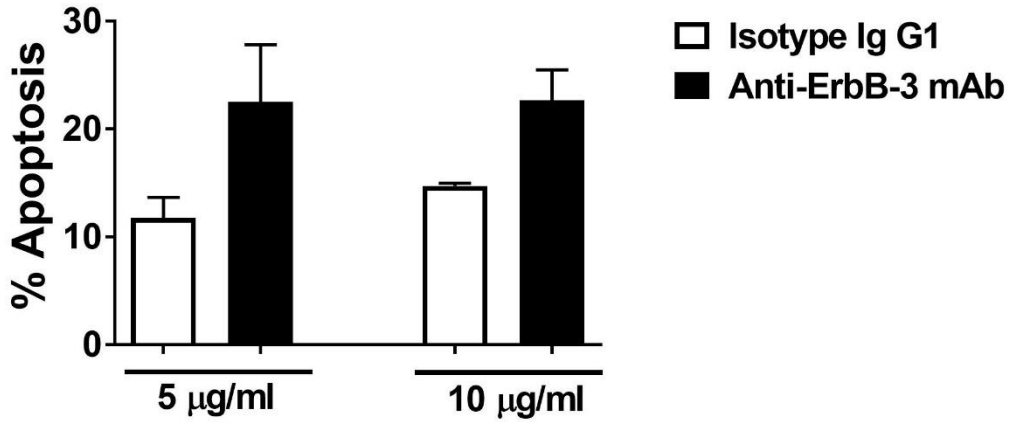


Figure 5.10. Neutralisation of ErbB3 accelerated neutrophil apoptosis. Neutrophils from healthy subjects were treated with anti-ErbB-3 mAb (and isotype IgG1 control) at 5 and 10 μg/ml for 5 hours followed by an assessment of apoptosis by light microscopy. Data represent 2 independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. Neutralization with ErbB-3 mAb (black bar) resulted in increased neutrophil apoptosis compared to isotype control (open bar), however no statistical test was performed as the experiment was performed twice. A total of 300 neutrophils were counted for each measurement by light microscopy. Data were kindly generated by an undergraduate project student, named Joseph Clarke.

5.2.3. Erbstatin analog and Tyrphostin AG825 reversed the effect of neutrophil pro-survival stimuli.

Pro-inflammatory factors that prolong neutrophil survival may undermine the therapeutic potential of ErbB inhibitors in resolving inflammation. It was therefore investigated whether Erbstatin analog and Tyrphostin AG825 can abrogate the effect of a number of known neutrophil pro-survival stimuli, such as cAMP, hypoxia, GMCSF and LPS, that convey a survival response via various signalling pathways (Dick et al., 2009, Klein et al., 2000, Krakstad et al., 2004, Martin et al., 2001, Vaughan et al., 2007, Walmsley et al., 2005, Wicks and Roberts, 2016). Intracellular cAMP levels are elevated by a number of inflammatory factors in inflammatory tissues and subsequently promote a neutrophil survival response via activation of protein kinase A dependent pathways (Krakstad et al.,

2004, Martin et al., 2001, Vaughan et al., 2007). To investigate the role of ErbB RTKs in cAMP-mediated survival, both Erbstatin analog and Tyrphostin AG825 were incubated with N^6-MB-cAMP (N^6-monobutyryl-cAMP, a cAMP analog) and neutrophil apoptosis measured at 6 and 20 hours. Both of these inhibitors significantly reversed the effect of the cAMP-mediated neutrophil survival response at 20 hours (p=0.032 and p=0.008, respectively) (**Figure 5.11 B and 5.12 B**), but not at 6 hours (p=0.22 and p>0.99) (**Figure 5.11 A and 5.12 A**). Tyrphostin AG825 not only overrode the cAMP-mediated neutrophil survival response but also enhanced apoptosis beyond control levels at 20 hours (**Figure 5.12 B**). Similar effects were also observed in neutrophils from COPD patients; neither Erbstatin analog nor Tyrphostin AG825 reversed cAMP-mediated neutrophil survival at 6 hours (p>0.99 and p>0.99) (**Figure 5.13 A&B**), while Tyrphostin AG825 reversed cAMP-mediated neutrophil survival response at 20 hours (p=0.023) (Erbstatin analog has not been tested) (**Figure 5.13 C**). Since cAMP-mediated signalling is very rapid, no effect of the ErbB inhibitors on the reversal of cAMP-mediated survival response at 6 hours may possibly be as a result of the rapid activation of cAMP signalling before ErbB inhibition takes place. To address this, neutrophils were pre-treated with Tyrphostin AG825 for 1 hour before the addition of N^6-MB-cAMP for a further 5 hours. Tyrphostin AG825 pre-treatment did not prevent cAMP-mediated survival at 6 hours (p=0.29) (**Figure 5.14 A**) and also significantly prevented cAMP-mediated survival at 20 hours (p=0.0006) (**Figure 5.14 B**). Tyrphostin AG825 pre-treatment for 4 hours prevented cAMP-mediated survival at 8 hours, although statistical tests were not performed as this represents 2 experiments (**Figure 5.14 C**). But this increased apoptosis with Tyrphostin AG825 could be a result of the initiation of Tyrphostin AG825-induced apoptosis by 4 hours before the cells get N^6-MB-cAMP.

Hypoxia, a characteristic feature of inflammation, is a known survival stimulus for neutrophils, acting via NF-kB (Walmsley et al., 2005). To test the effect of Erbstatin analog and Tyrphostin AG825 on the hypoxia-mediated neutrophil survival, neutrophils were co-cultured with these inhibitors under normoxia (Po_2≈19 KPa) and hypoxia (Po_2≈3

KPa) for 20 hours followed by an assessment of apoptosis by light microscopy. Neither Erbstatin analog nor Tyrphostin AG825 prevented the hypoxic neutrophil survival, however, Tyrphostin AG825 showed a trend on reversing the survival response (p=0.165 and p=0.0795). (**Figure 5.15 A&B**).

GMCSF, a key neutrophil survival factor, binds to its surface receptor and activates the PI3K pathway leading to promotion of neutrophil survival (Yasui et al., 2002). LPS is also a key survival factor, and LPS-driven neutrophil survival is instigated by binding of LPS to TLR4, conveying the apoptosis inhibition signals via MAPK, PI3K and NF-kB pathways (Dick et al., 2009, Sabroe et al., 2002, Sabroe et al., 2003, Ward et al., 1999, Ward et al., 2005, Ward et al., 2004). Erbstatin analog treatment at two different concentrations was found to reverse LPS-mediated neutrophil survival at 6 hours, as determined by both light microscopy ($p<0.0001$) (**Figure 5.16 A**) and flow cytometry ($p=0.0133$) (**Figure 5.16 B**). Erbstatin analog not only reversed the LPS-mediated pro-survival response but also enhanced apoptosis beyond baseline controls (**Figure 5.16 A&B**). Similarly, Erbstatin analog also reversed the GMCSF-mediated pro-survival effects in healthy subjects, as determined by light microscopy ($p<0.0001$) (**Figure 5.17 A**) and flow cytometry ($p=0.006$) (**Figure 5.17 B**). It also enhanced apoptosis beyond baseline controls. To investigate whether ErbB inhibitors can override the pro-survival effects of GMCSF in neutrophils from patients with systemic inflammation, neutrophils were also isolated from patients with COPD and age-matched healthy subjects. Both Erbstatin analog (**Figure 5.18 A&B**) and Tyrphostin AG825 (**Figure 5.19 A&B**) dose-dependently overrode the pro-survival effect of GMCSF both in COPD and age-matched healthy control subjects, at 6 and 20 hours [Erbstatin analog: $p<0.05$ (COPD), $p<0.05$ (HC) and $p<0.0001$ (COPD), $p<0.0001$ (HC) for 6 and 20 hours; Tyrphostin AG825: $p<0.0$ (COPD), $p=0.243$ (HC) and $p<0.0001$ (COPD), $p<0.001$ (HC) for 6 and 20 hours].

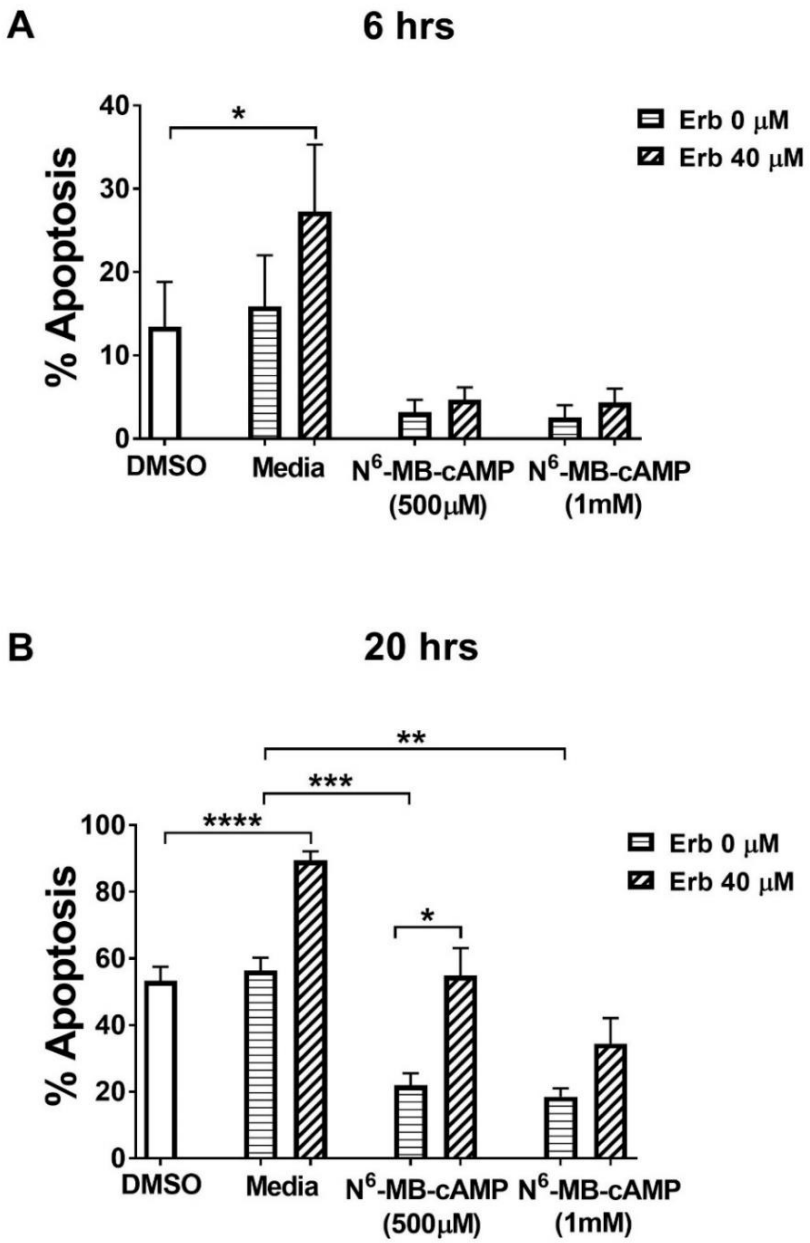


Figure 5.11. Erbstatin analog prevented cAMP-mediated neutrophil survival. Neutrophils from healthy subjects were treated with or without Erbstatin analog [Erb, 0, 40 μM] in the presence or absence of N^6-MB-cAMP [500 μM, 1 mM] for 6 (A) or 20 (B) hours followed by an assessment of apoptosis by light microscopy. Data represent 4 independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. Media is the negative control; DMSO is the vehicle control for Erbstatin analog. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *p<0.05, **p<0.01, ***p<0.001, ****P<0.0001

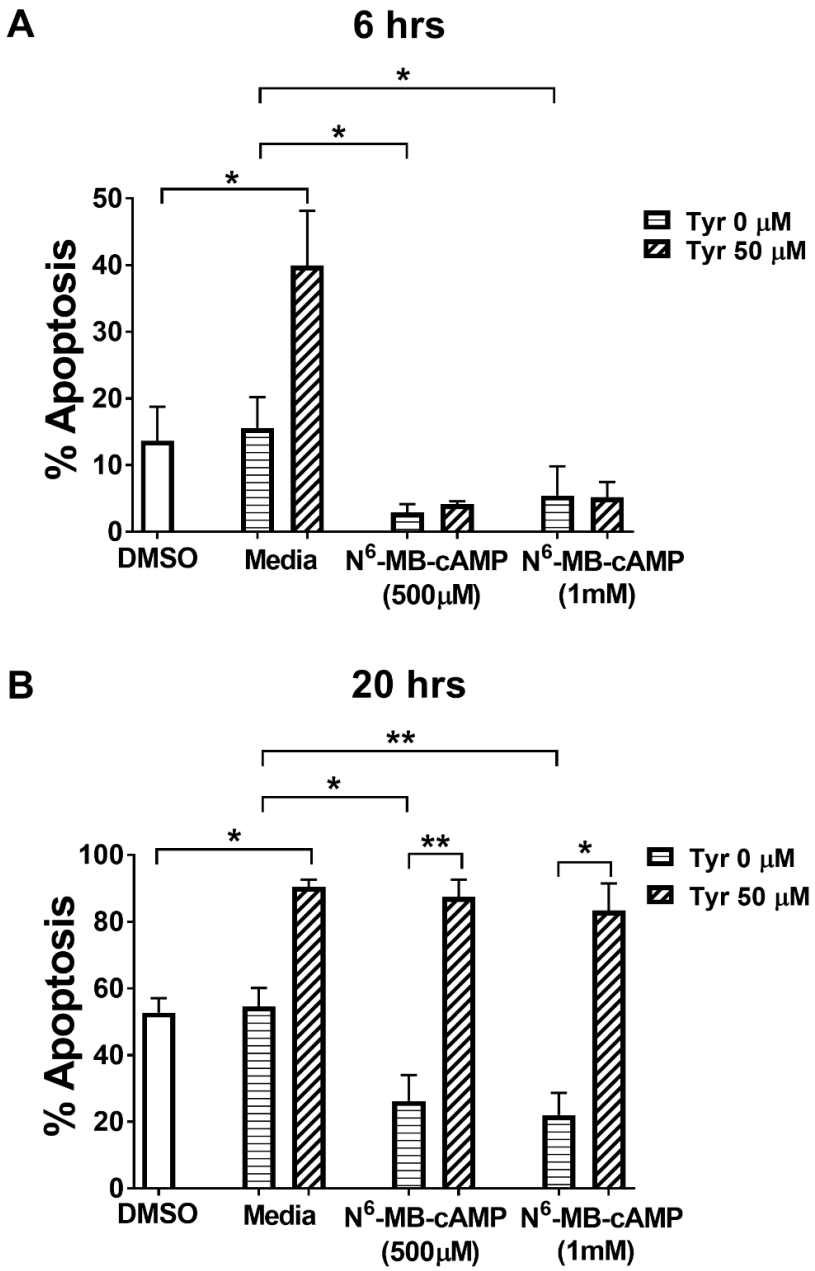


Figure 5.12. Tyrphostin AG825 prevented cAMP-mediated neutrophil survival. Neutrophils from healthy subjects were treated with or without Tyrphostin AG825 [Tyr, 0, 50 μM] in the presence or absence of N^6-MB-cAMP [500 μM, 1 mM] for 6 (A) or 20 (B) hours followed by assessment of apoptosis by light microscopy. Data represent 4 independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. Media is the negative control; DMSO is the vehicle control for Tyrphostin AG825. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *p<0.05, **p<0.01.

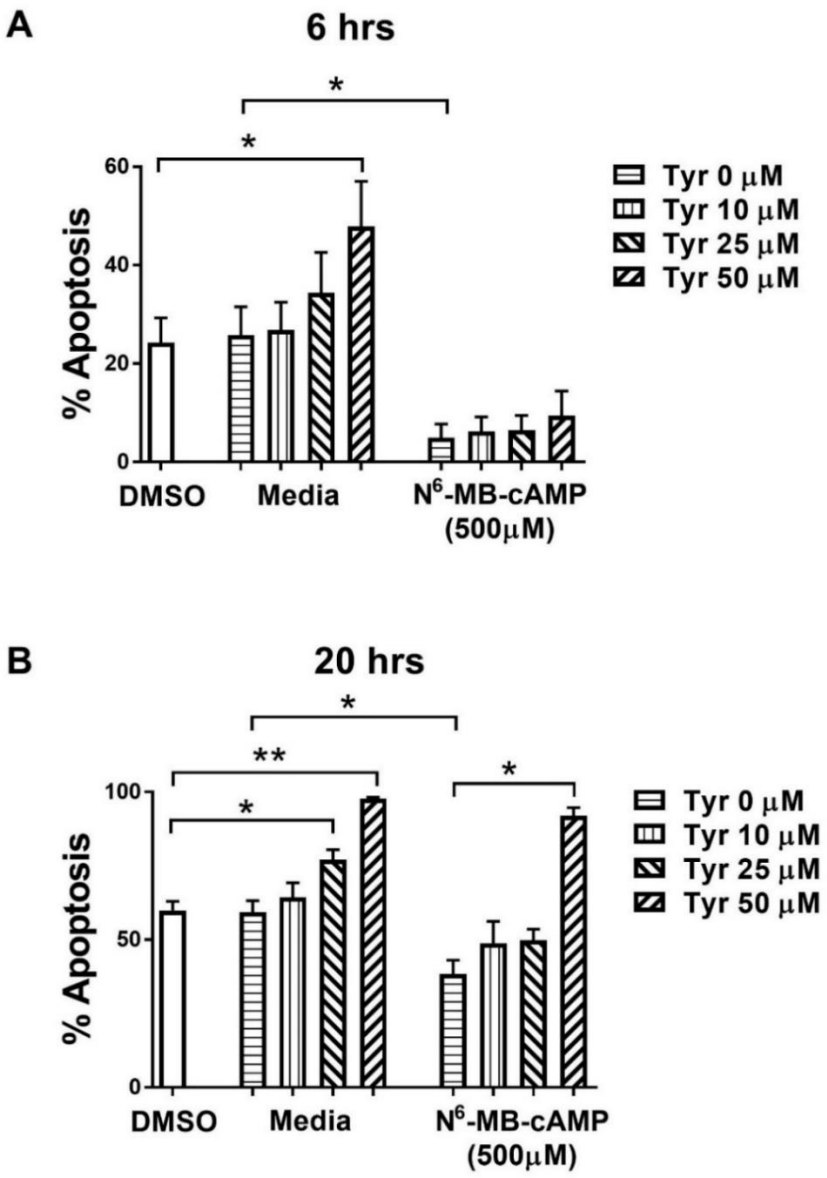


Figure 5.13. Tyrphostin AG825 prevented cAMP-mediated survival in COPD neutrophils. Neutrophils from COPD patients were treated with or without Tyrphostin AG825 [Tyr, 0, 10, 25 and 50 μM] in the presence or absence of N^6-MB-cAMP [500 μM] for 6 (A) or 20 (B) hours followed by an assessment of apoptosis by light microscopy. Data represent 4 independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. Media is the negative control; DMSO is the vehicle control for Tyrphostin AG825. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *$p<0.05$, **$p<0.01$.

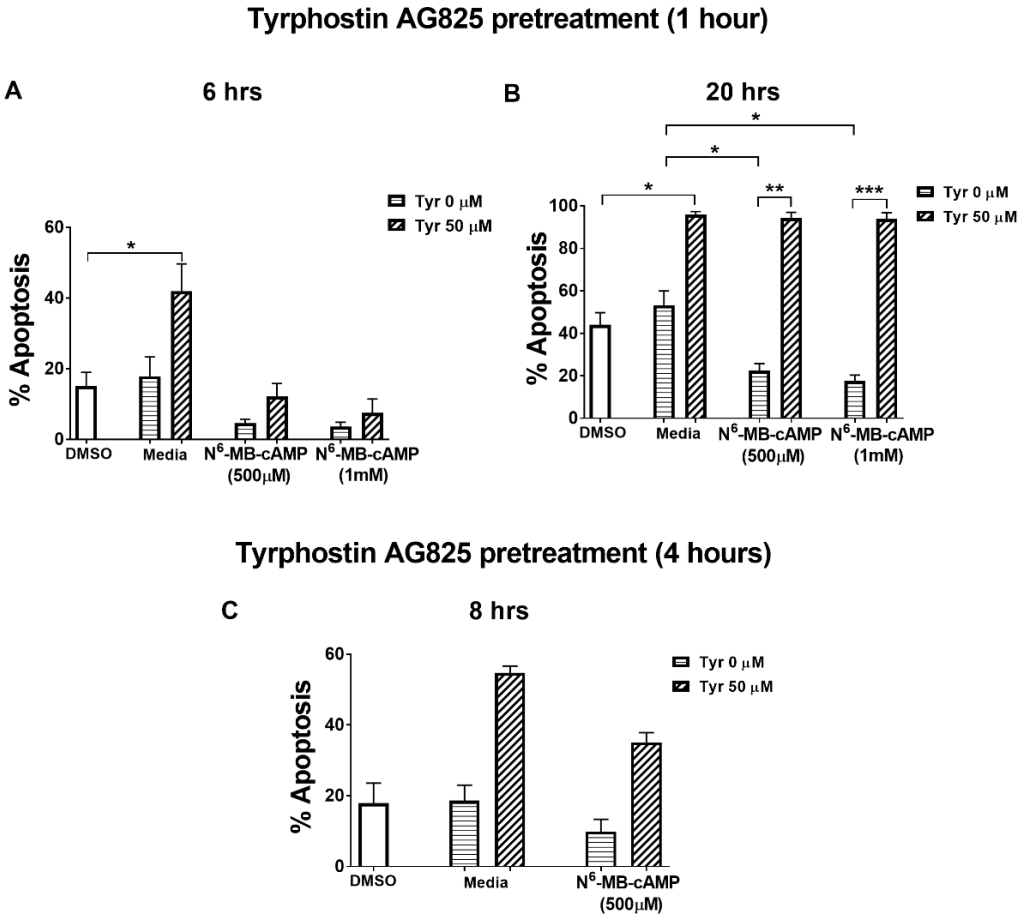


Figure 5.14. Tyrphostin AG825 pre-treatment prevented cAMP-mediated survival. Neutrophils from healthy subjects were pre-treated with or without Tyrphostin AG825 [Tyr, 0, 50 μM] for 1 (A, B) or 4 (C) hours followed by the addition of N^6-MB-cAMP [500 μM, 1 mM (A, B) or 500 μM (C)]. Apoptosis was assessed at 6 (A) or 20 (B) or 8 (C) hours by light microscopy. Data represent 4 (A, B) or 2 (C) independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. Media is the negative control; DMSO is the vehicle control for Tyrphostin AG825. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *p<0.05, **p<0.01, ***p<0.001.

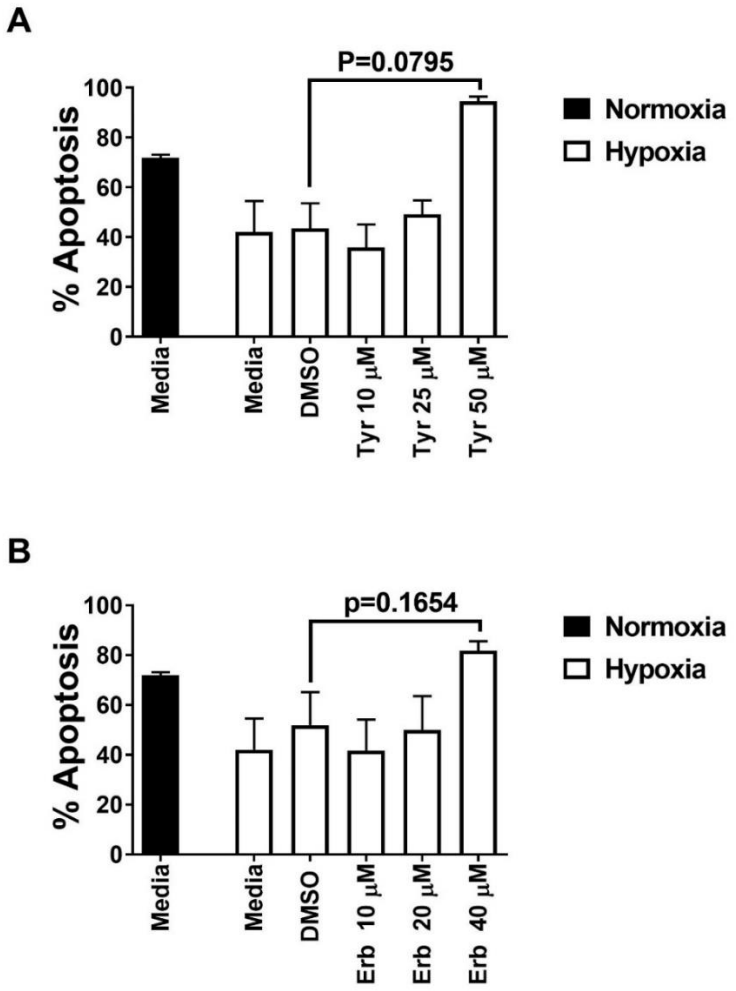


Figure 5.15. Tyrphostin AG825 and Erbstatin analog did not prevent hypoxic neutrophil survival. Neutrophils from healthy subjects were cultured with or without Tyrphostin AG825 [Tyr, 0, 10, 25, 50 µM] (A) or Erbstatin analog [Erb, 0, 10, 20, 40 µM] (B) for 20 hours under normoxic [pO_2≈19 KPa] and hypoxic [pO_2≈3 KPa] conditions. The constitutive and hypoxic rates of neutrophil apoptosis were assessed by light microscopy. Data represent 3 (A, B) independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. Media is the negative control; DMSO is the vehicle control for Tyrphostin AG825 (A) or Erbstatin analog (B). A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Bonferroni's post-hoc test and p-values are stated in the figure.

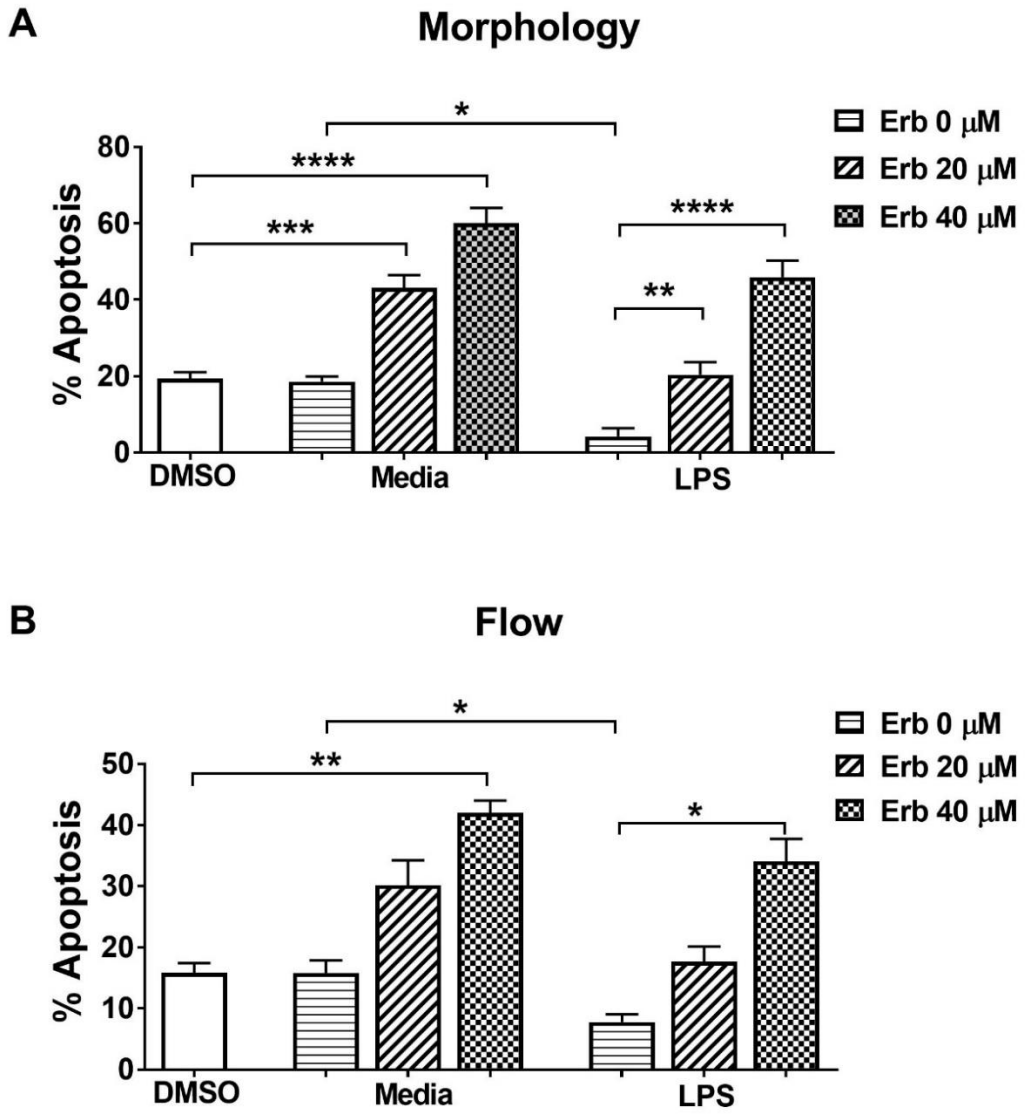


Figure 5.16. Erbstatin analog prevented LPS-mediated neutrophils survival. Neutrophils from healthy subjects were treated with Erbstatin analog [Erb, 0, 20 and 40 μM] in the presence or absence of LPS [*E. coli* LPS, 1 μg/ml] for 6 hours. Apoptosis was assessed by light microscopy (A) and flow cytometry by annexin V-PE/Topro-3 staining (B). Data represent 4 (A, B) independent experiments with duplicate measurements under each condition, and the values are expressed as mean percent apoptosis ± SEM. Media is the negative control; DMSO is the vehicle control for Erbstatin analog. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *p<0.05, **p<0.01, ***p<0.001, ****P<0.0001.

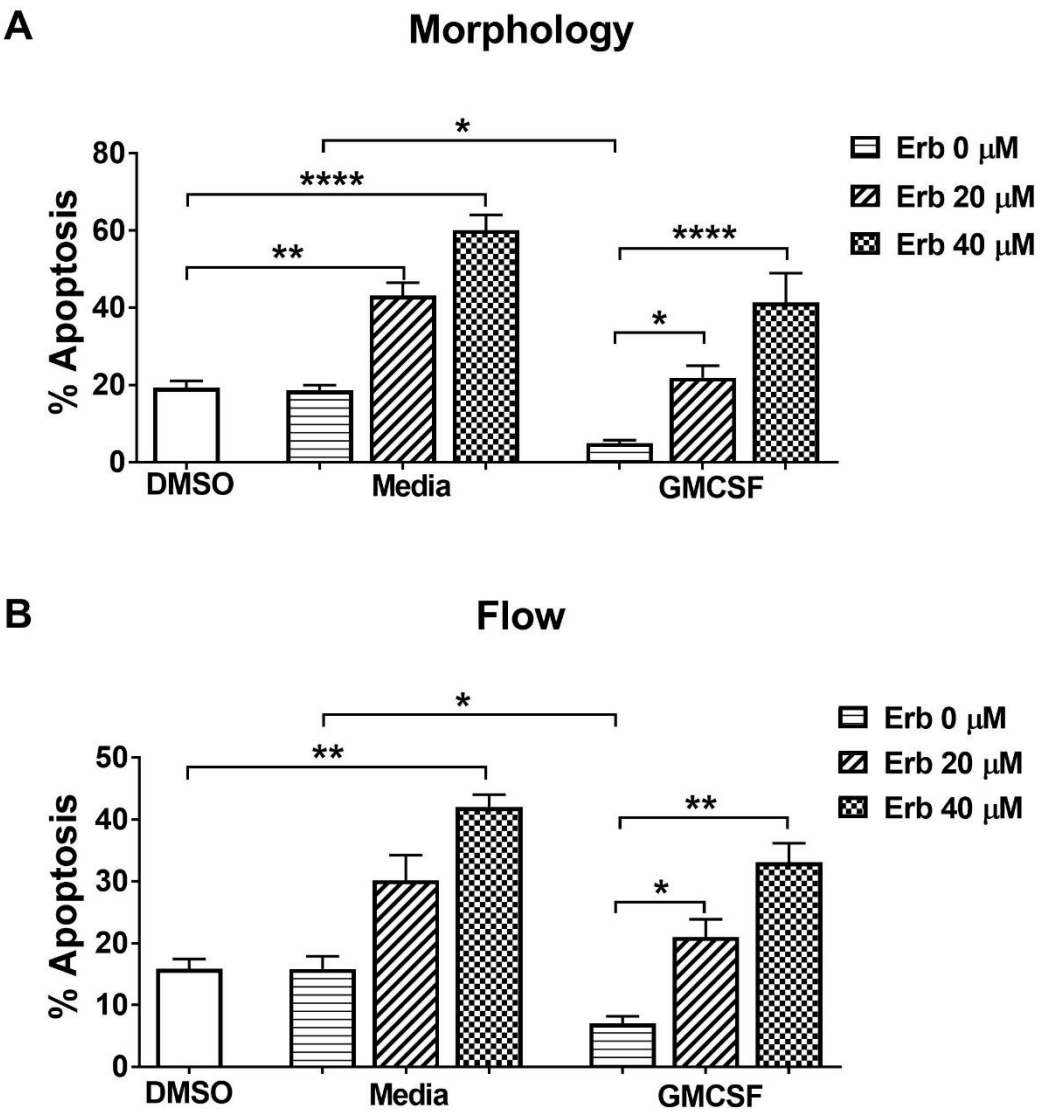


Figure 5.17. Erbstatin analog prevented GMCSF-mediated neutrophils survival. Neutrophils from healthy subjects were treated with Erbstatin analog [0, 20 and 40 μM] in the presence or absence of GMCSF [50 U/ml] for 6 hours followed by assessment of apoptosis by light microscopy (A) or flow cytometry (B) by annexin V-PE/Topro-3 staining. Data represent 4 (A, B) independent experiments with duplicate measurements under each condition, and the values are expressed as mean ± SEM. Media is the negative control; DMSO is the vehicle control for Erbstatin analog. A total of 300 neutrophils were counted for each measurement by light microscopy. The statistical significance was calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *p≤0.05, **p≤0.01, ***p≤0.001, ****P≤0.0001.

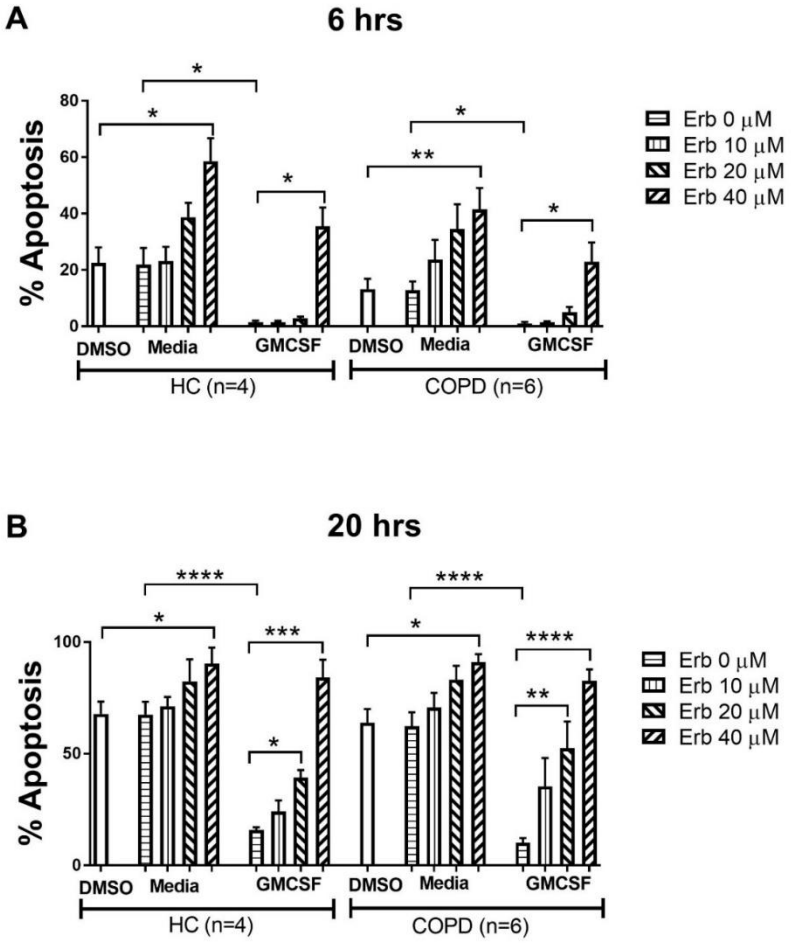


Figure 5.18. Erbstatin analog prevented GMCSF-mediated neutrophil survival in COPD patients and age-matched healthy subjects. Neutrophils from COPD patients (COPD) and age matched healthy subjects (HC) were treated with Erbstatin analog [Erb, 0, 10, 20 and 40 μM] with or without GMCSF [50 U/ml] for 6 (A) or 20 (B) hours, followed by an assessment of apoptosis by light microscopy. Experiments were performed with duplicate measurements in each condition, and the values are expressed as mean percent apoptosis ± SEM, n=6 (COPD), 4 (HC). Media is the negative control; DMSO is the vehicle control for Erbstatin analog. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *p<0.05, **p<0.01, ***p<0.001, ****P<0.0001.

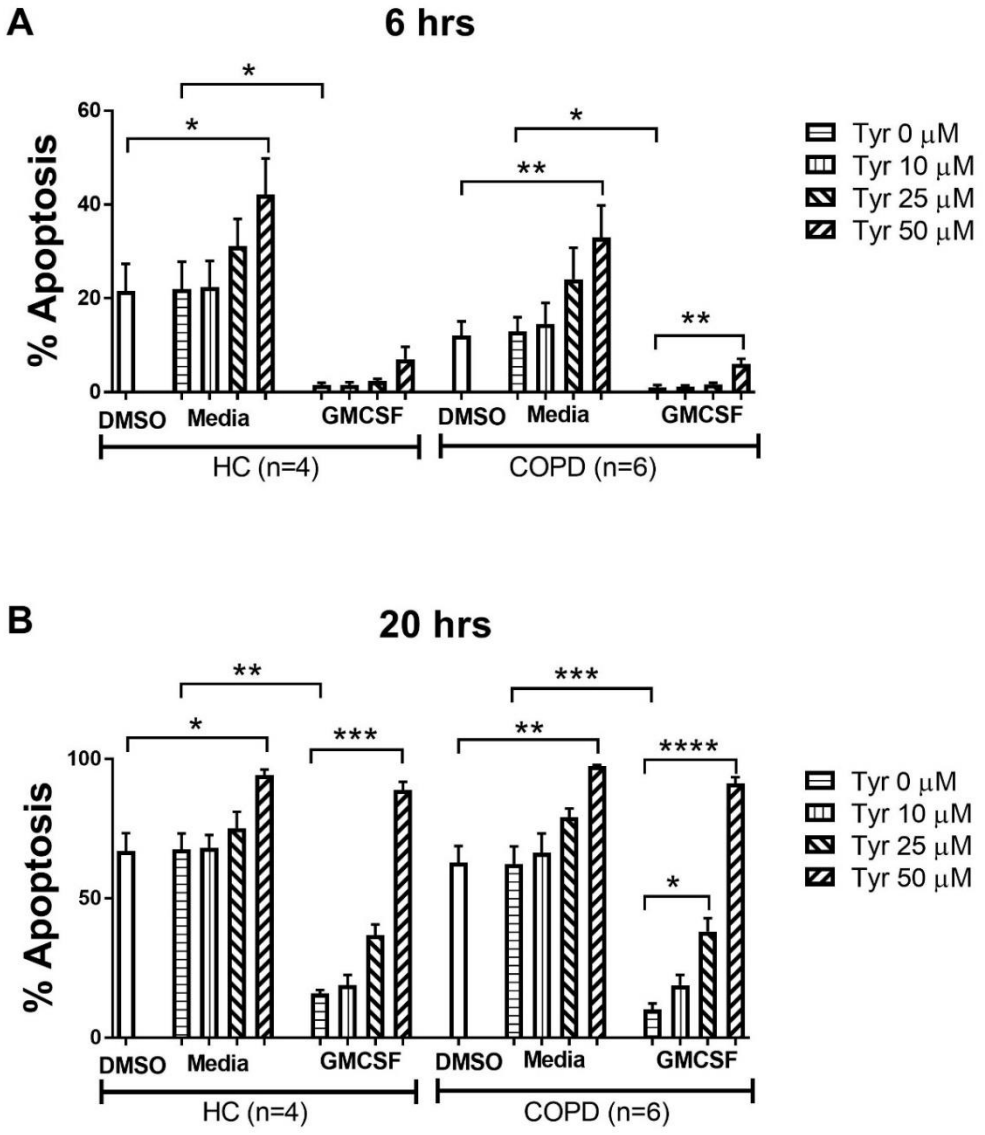


Figure 5.19. Tyrphostin AG825 prevented GMCSF-mediated neutrophil survival in COPD patients and age-matched healthy subjects. Neutrophils from COPD patients (COPD) and age matched healthy subjects (HC) were treated with AG825 [Tyr, 0, 10, 25 and 50 μM] with or without GMCSF [50 U/ml] for 6 (A) or 20 (B) hours, followed by an assessment of apoptosis by light microscopy. Experiments were performed with duplicate measurements in each condition, and the values presented in each condition are expressed as mean percent apoptosis ± SEM, n=6 (COPD), 4 (HC). Media is the negative control; DMSO is the vehicle control for Tyrphostin AG825. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significances were calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *p<0.05, **p<0.01, ***p<0.001, ****P<0.0001.

5.2.4. Tyrphostin AG825 reduced PI3K/AKT activation and Mcl-1 level.

The pro-survival effect of GMCSF is mediated via activation of the PI3K/AKT pathway leading to the stabilisation of the anti-apoptotic Bcl-2 family protein, Mcl-1 (Derouet et al., 2004, Klein et al., 2000, Moulding et al., 1998). To investigate the mechanism of how Tyrphostin AG825 reverses the GMCSF-mediated survival response, AKT-phosphorylation, as a measure of PI3K activation, was assessed. Tyrphostin AG825 pre-treatment for 1 hour was found to reduce GMCSF-induced AKT-phosphorylation following 15 and 30 minutes of GMCSF treatment ($p<0.05$) (**Figure 5.20 A**). Mcl-1 plays critical roles in the regulation of neutrophil apoptosis/survival and neutrophil survival has been shown to correlate with the level of Mcl-1 (Michels et al., 2005, Moulding et al., 1998). Therefore, to identify potential downstream mechanisms of Tyrphostin AG825 driven apoptosis, neutrophils were pre-treated with Tyrphostin AG825 for 1 hour in the presence or absence of pan-caspase inhibitor QVD followed by the addition of GMCSF, and Mcl-1 levels were measured by western blotting at 8 hours, the timepoint at which Mcl-1 degradation mechanism in the regulation of neutrophil apoptosis was reported to take place as an upstream event of caspase activation (Wardle et al., 2011). Tyrphostin AG825 was found to reduce constitutive and GMCSF-induced levels of Mcl-1 (**Figure 5.20 B**), suggesting that Tyrphostin AG825 induced neutrophil apoptosis is mediated via degradation of Mcl-1. Furthermore, GMCSF-induced Mcl-1 levels were reduced with Tyrphostin AG825 treatment even in the presence of QVD (**Figure 5.20 B**), suggesting that Tyrphostin AG825-induced Mcl-1 degradation at this timepoint takes place as upstream component rather than a substrate of caspases.

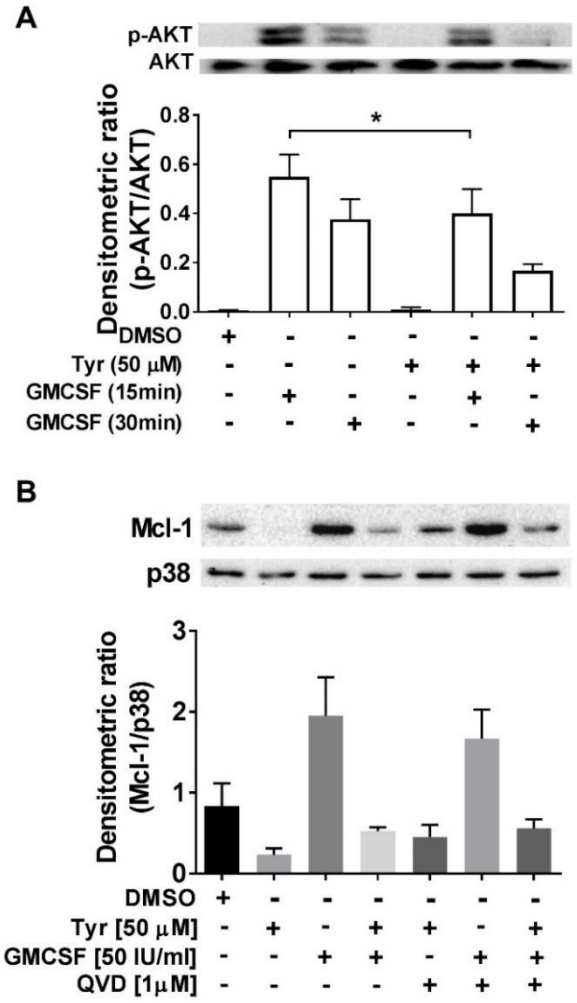


Figure 5.20. Tyrphostin AG825 prevented GMCSF-induced AKT-phosphorylation and destabilise Mcl-1. (A) Neutrophils from healthy subjects were pre-treated with Tyrphostin AG825 [Tyr, 0, 50 µM] for 1 hour followed by the addition of GMCSF [50 U/ml] for 15 and 30 minutes. (B) Neutrophils were pre-treated with Tyrphostin AG825 [Tyr, 0, 50 µM] in the presence or absence of Q-VD-OPh [QVD,1 µM] for 1 hour followed by the addition of GMCSF [50 U/ml] for a further 7 hours. Cell lysates were made for western blot analysis, probing for p-AKT (A) or Mcl-1 (B) and also loading controls, AKT (A) or p38 (B). Immunoblot images representing the levels of p-AKT and AKT (A), or Mcl-1 and p38 are shown. The densitometric ratios [p-AKT/AKT (A) or Mcl-1/p38 (B)] are presented in the graphs. Data represent 3 (A, B) independent experiments, and the values are expressed as mean ± SEM. DMSO is the vehicle control for Tyrphostin AG825. Statistical significance was calculated by one way ANOVA followed by Bonferroni's post-hoc test and presented as *$p<0.05$.

5.2.5. Tyrphostin AG825 reduced neutrophil number at the site of inflammation in a zebrafish tail injury model.

In order to examine the effect of Tyrphostin AG825 on neutrophilic inflammation in a complex organism, a zebrafish model was used. More specifically, the effect of Tyrphostin AG825 on the number of neutrophils at the site of inflammation was studied in a well-characterized mpx:GFP zebrafish tailfin injury *in vivo* model (Renshaw et al., 2006). The kinetics of neutrophil recruitment at the site of inflammation was visualised following tail transection, and neutrophil numbers were found to increase until 6 hours post injury (hpi) followed by a gradual reduction at 24 hpi as inflammation resolved (**Figure 5.21 A&B**). To assess the effect of Tyrphostin AG825 on inflammatory neutrophils, fish larvae were pre-treated with Tyrphostin AG825 for 24 hours before they underwent tail transection and the number of neutrophils at the site of inflammation was counted at 4 and 8 hpi. Tyrphostin AG825 pre-treatment for 24 hours resulted in significant reduction of the neutrophil number at 4 and 8 hpi ($p=0.0117$ and $p=0.0108$) (**Figure 5.21C**). However, the number of total neutrophils across the whole zebrafish body was unaffected following Tyrphostin AG825 treatment for 24 hours ($p=0.639$) (**Figure 5.22 A&B**), suggesting that inflammatory neutrophils are more sensitive to the effect of Tyrphostin AG825.

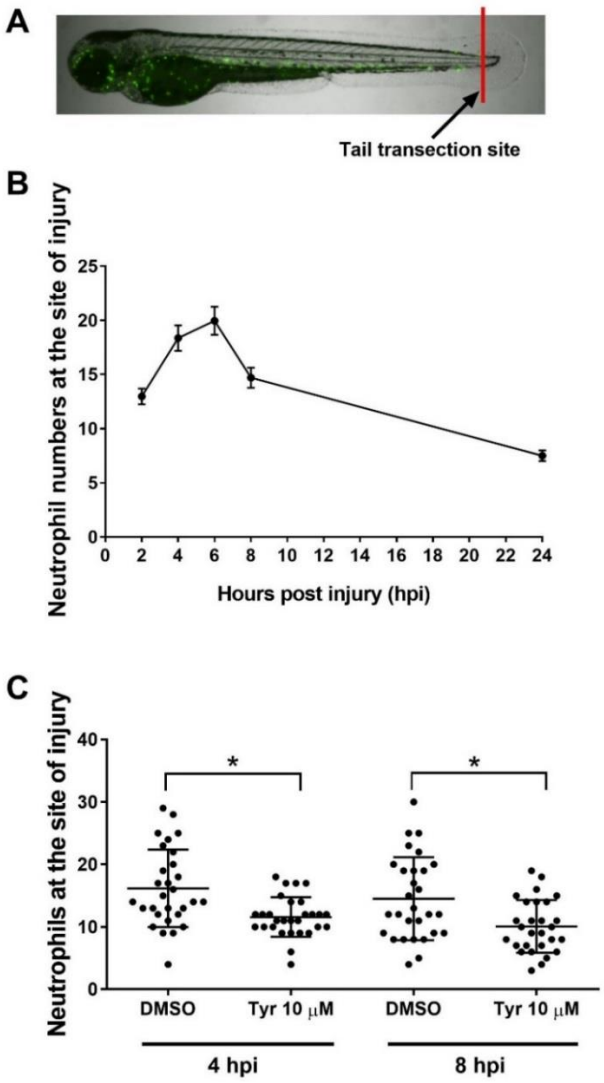


Figure 5.21. Tyrphostin AG825 reduced number of neutrophil at the site of injury in zebrafish tail injury model. Tails of Tg(mpx:GFP)i114 zebrafish larvae were transected to induce inflammatory responses and GFP-positive neutrophils were visualised and counted by fluorescence microscopy. (A) A picture of a representative fish larva indicating the site of tail transection (red line) is shown. (B) Tails of 3 days post fertilized (dpf) larvae were transected and neutrophil numbers were counted at 2, 4, 6, 8 and 24 hours post injury (hpi). (C) 2 dpf larvae were pre-treated with or without Tyrphostin AG825 [Tyr,10 μM] for 24 hours before underwent tail transection and neutrophil numbers counted at the site of injury at 4 and 8 hpi. Data represent at least 30 fishes under each condition performed as 3 independent experiments and the values are expressed as mean ± SEM. DMSO is the vehicle control for Tyrphostin AG825. Statistical significances were calculated by one way ANOVA (with Bonferroni post-hoc test) and presented as *$p<0.05$.

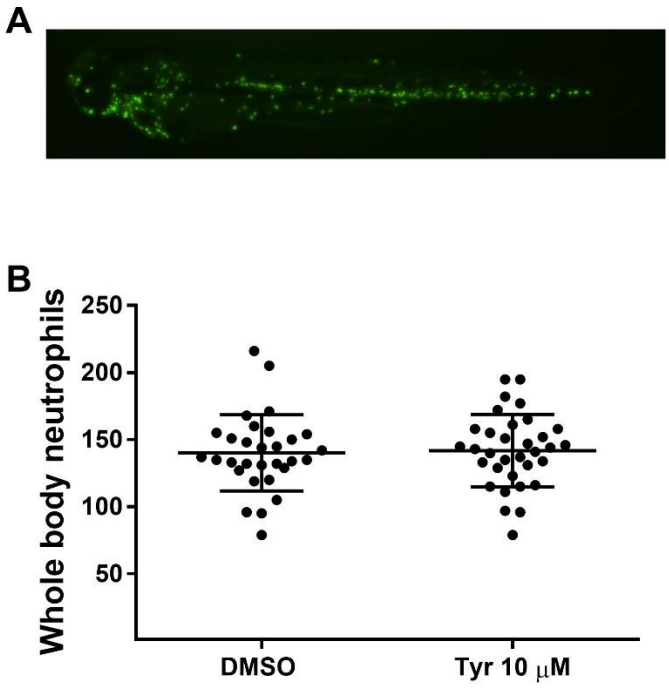


Figure 5.22. Tyrphostin AG825 did not affect the number of neutrophils across the whole body. 2 dpf larvae of Tg (mpx:GFP)i114 zebrafish were treated with or without Tyrphostin AG825 [Tyr, 10 μM] for 24 hours followed by mounting the fish in 0.8% low melting point agarose. GFP-positive neutrophils were then counted from image taken by a fluorescent microscope. (A) GFP-positive neutrophils across the whole body of a representative larva are shown. (B) A graph representing the neutrophil numbers across the whole body following DMSO (control) or Tyrphostin AG825 [10 μM] treatment is shown. Data represents at least 30 fishes under each condition performed as 3 independent experiments and the values are expressed as mean ± SEM. DMSO is the vehicle control for Tyrphostin AG825. Statistical significance was calculated by a non-parametric t-test (Mann-Whitney U test).

5.2.6. Tyrphostin AG825 increased neutrophil apoptosis and efferocytosis in an acute lung injury murine model.

In order to investigate the effect of ErbB inhibition on neutrophil apoptosis *in vivo*, a murine model of acute inflammation was adopted. To specifically address the ability of Tyrphostin AG825 to accelerate neutrophil apoptosis in the mammalian lung, an LPS-induced murine model of acute inflammation was used (Asti et al., 2000). C57BL/6 mice were nebulised with LPS to induce neutrophilic inflammation (Asti et al., 2000, Faffe et

al., 2000, Thompson et al., 2013), followed by an immediate intraperitoneal injection with 20 mg/kg Tyrphostin AG825 (Tyr) or DMSO (control). Mice were lavaged after 48 hours and the cellular component was cytocentrifuged onto microscope slides. The assessment of neutrophil apoptosis and macrophage-mediated efferocytosis of apoptotic bodies was carried out by light microscopy and flow cytometry. Tyrphostin AG825 treatment was found to significantly increase neutrophil apoptosis compared to control (p=0.005) (**Figure 5.23 A**). *In vivo* visualising free apoptotic neutrophils is a rare event because of a continuous efferocytosis process by residential macrophages. Therefore, neutrophil apoptosis was also estimated by the summation of the free apoptotic neutrophils along with the apoptotic neutrophils visualised inside of macrophages. A similar and significant increase in neutrophil apoptosis was observed in the Tyrphostin AG825 treatment group (p=0.010) (**Figure 5.23 B**). However, BAL neutrophil apoptosis as assessed by flow cytometry (by annexin-V/Topro-3 staining) was not significantly altered by Tyrphostin AG825 (p=0.819); however, the data were variable because of low apoptotic events (**Figure 5.23 C**). Tyrphostin AG825 treatment also enhanced macrophage-mediated efferocytosis compared to control, as assessed by calculating the percent of macrophages containing apoptotic bodies out of total macrophages (p=0.0008) (**Figure 5.24 A&B**). Tyrphostin AG825 treatment did not alter the percent of neutrophils or macrophages (**Figure 5.25 A-C**). Neither neutrophil count (**Figure 5.23 D-F**) nor total cell count (**Figure 5.25 G-H**) was altered with Tyrphostin AG825 treatment compared to control.

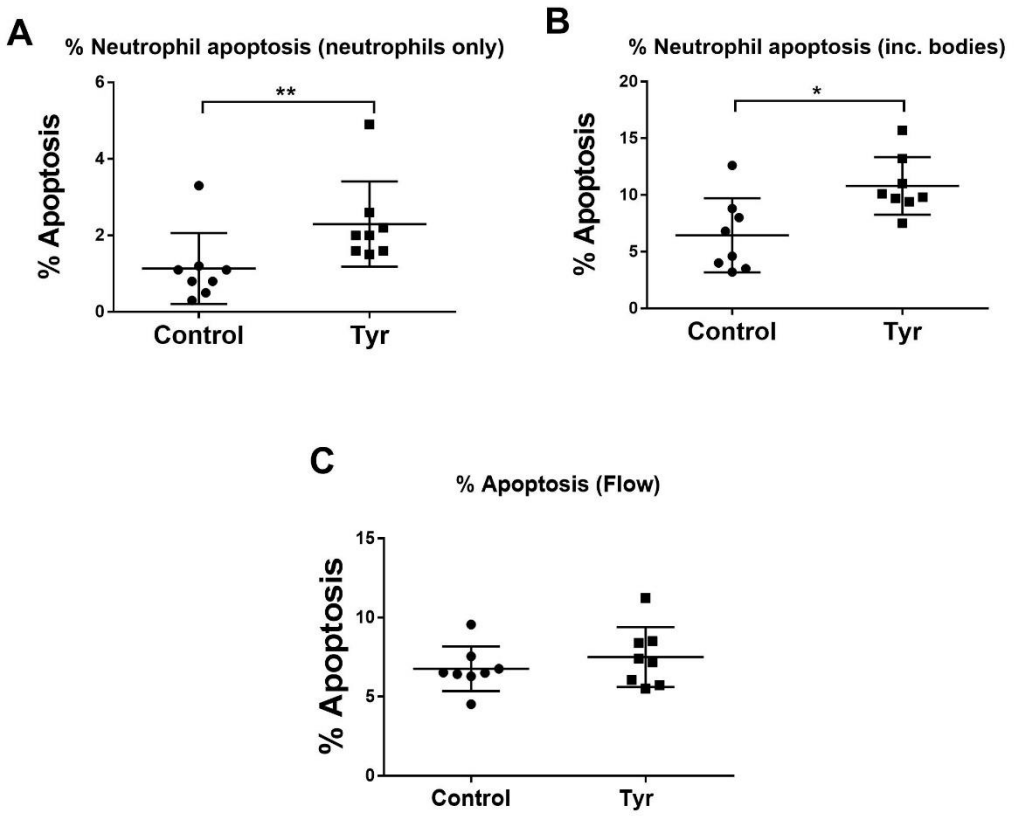


Figure 5.23. Tyrphostin AG825 promoted neutrophil apoptosis in an LPS-induced acute lung inflammation model. C57BL/6 mice were nebulised with LPS (3 mg per group) followed by an immediate intraperitoneal injection with 20 mg/kg Tyrphostin AG825 (Tyr, n=8) or 10% DMSO (control, n=8). BAL samples were then collected at 48 hours. Neutrophil apoptosis in the BAL samples was calculated by oil immersion light microscopy (A, B) or by flow cytometry by annnexin V-PE/Topro-3 staining (C). Light microscopy mediated apoptosis assessment were calculated in terms of the free apoptotic neutrophils (A) or by the by the summation of the proportion of the isolated apoptotic neutrophils with the inclusions of apoptotic bodies within macrophages (B). The data are expressed as mean ± SEM. A total of 300 neutrophils were counted for each measurement by light microscopy. The statistical significances were calculated by a non-parametric t-test (Mann–Whitney U test), and presented as *p<0.05, **p<0.01.

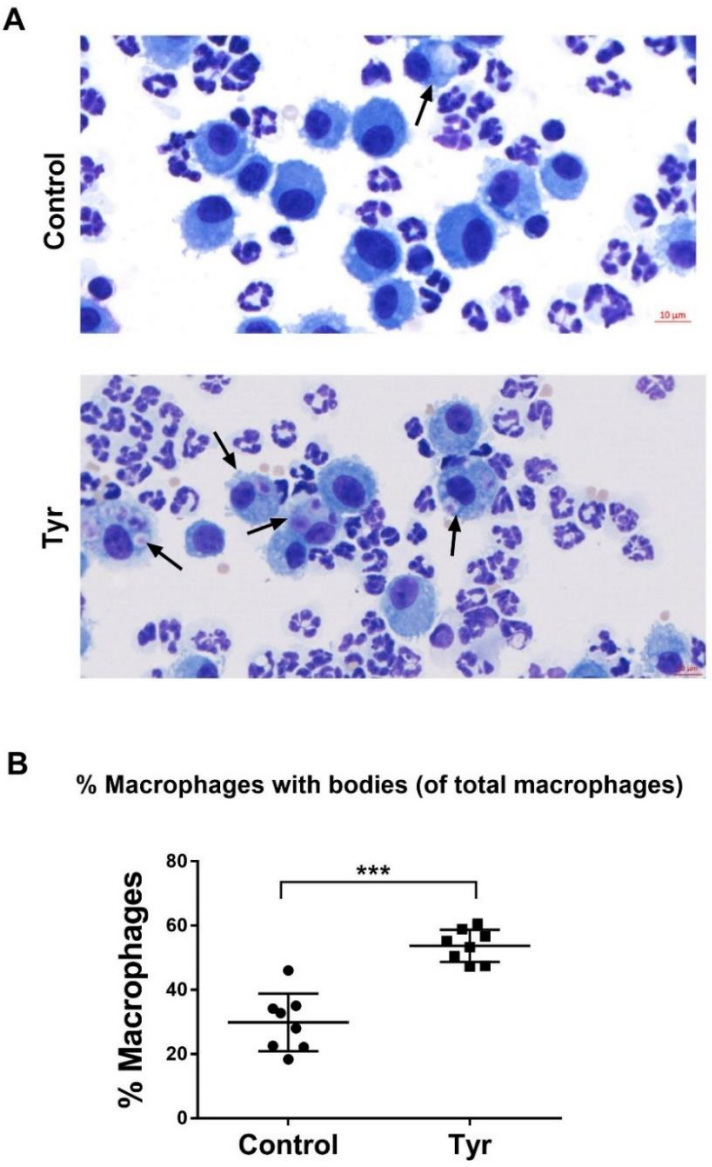


Figure 5.24. Tyrphostin AG825 increased macrophage efferocytosis in an LPS-induced acute lung inflammation model. C57BL/6 mice were nebulised with LPS (3 mg per group) followed by an immediate intraperitoneal injection with 20 mg/kg Tyrphostin AG825 (Tyr, n=8) or 10% DMSO (control, n=8). BAL samples were then collected at 48 hours. Alveolar macrophage mediated efferocytosis of apoptotic neutrophils were calculated by oil immersion light microscopy. (A) Alveolar macrophages engulfed apoptotic bodies are indicated by black arrows. (B) A graphical representation showing the percent macrophages with 1 or more apoptotic body inclusions (of total macrophages) are shown. The data are expressed as mean ± SEM. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significance was calculated by a non-parametric t-test (Mann–Whitney U te st), and presented as ***p<0.001.

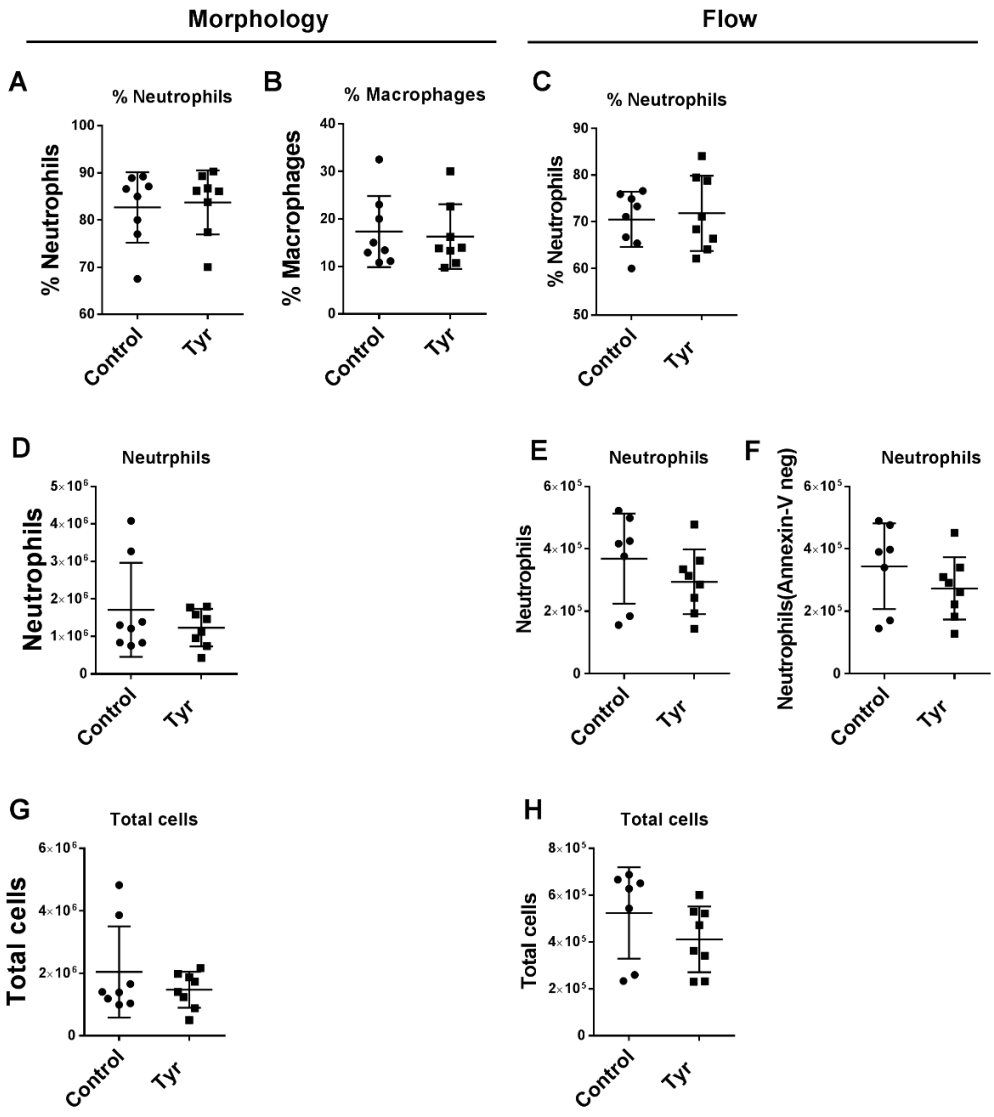


Figure 5.25. Tyrphostin AG825 increased neutrophil apoptosis in an LPS-induced acute lung inflammation model. C57BL/6 mice were nebulised with LPS (3 mg per group) followed by an immediate intraperitoneal injection with 20 mg/kg Tyrphostin AG825 (Tyr, n=8) or 10% DMSO (control, n=8). BAL samples were then collected at 48 hours. Differential counts of BAL cells were calculated by oil immersion light microscopy and flow cytometry. (A & B) % neutrophils and % macrophages by light microscopy, respectively. (C) % neutrophils by flow cytometry based on annexin V-PE/Topro-3 positivity. (D & E) Neutrophil numbers assessed by light microscopy and flow cytometry, respectively. (F) The number of healthy neutrophils (annexin-V negative) as assessed by flow cytometry. (G & H) The total number of cells as determined by a haemocytometer and by flow cytometry, respectively. The data are expressed as mean ± SEM. A total of 300 neutrophils were counted for each measurement by light microscopy. Statistical significance was calculated by a non-parametric t-test (Mann–Whitney U test).

5.3. Discussion

Apoptosis plays a vital role in the inflammation resolution process, mediating functional shut down of neutrophils followed by clearance by residential macrophages (El Kebir and Filep, 2013b, Savill et al., 1989b, Whyte et al., 1999). Since delayed neutrophil apoptosis has been implicated in a number of chronic inflammatory diseases, defining neutrophil apoptosis pathways and therefore, identifying novel therapeutically sensitive targets is of great importance for the development of therapeutics for inflammatory diseases which is currently an unmet need. In this chapter, the ErbB family of RTKs are shown to be important regulators of neutrophil survival. Inhibition of ErbB1 and ErbB2, by selective small molecule inhibitors Erbstatin analog and Tyrphostin AG825, resulted in enhanced neutrophil apoptosis *in vitro* and *in vivo*, and reduced neutrophil number at the site of inflammation *in vivo*.

The ErbB family RTKs are among the most studied cell signalling families in biology (Lemmon and Schlessinger, 2010). RTKs consist of an extracellular ligand binding domain, a hydrophobic transmembrane segment and an intracellular kinase domain (Prenzel et al., 2001), although there are some exceptions (discussed below in this paragraph). Ligand binding to the extracellular domain results in receptor homo- or hetero-dimerisation subsequently leading to autophosphorylation of tyrosine residues at the cytoplasmic domain which leads to activation of the kinases and downstream signalling (Roskoski, 2014). The phosphorylated tyrosine residues serve as the docking sites for numerous signalling molecules involved in multiple signalling pathways, and therefore activation of ErbB receptors transduces the signal to heterologous cellular pathways (Hynes et al., 2001). Among the four ErbB family kinases, ErbB1 and ErbB4 are considered as classical RTKs, whereas ErbB2 has no known ligand binding domain and ErbB3 is kinase impaired (Casalini et al., 2004, Prenzel et al., 2001, Roskoski, 2014). Among the four members, all kinases can form heterodimers with their ErbB partners, and the ErbB heterodimers generate more potent mitogenic signals than homodimers

(Maruyama, 2014). Since ErbB2 does not require a ligand, ErbB2 homodimers are constitutively active (Fan et al., 2008).

Over-expression and dysregulated activation of these kinases are associated with the development of multiple cancers, including breast, neck, lung and brain cancer (Roskoski, 2014, Yarden and Pines, 2012). Development of cancer as a result of activation of ErbB1 and ErbB2 kinases is mediated via multiple mechanisms such as overexpression, mutation, deletion and autocrine ligand-receptor interaction (Hynes and MacDonald, 2009). The oncogenic activity of ErbB2 is mainly driven by overexpression or by making interaction with ErbB1 and ErbB3 (Hynes and MacDonald, 2009). Overexpression of ErbB2 is associated with 30% of human breast cancer (Slamon et al., 1987, Slamon et al., 1989) and many other cancers (Roskoski, 2014), and is also associated with poor clinical outcomes (Berchuck et al., 1990, Slamon et al., 1987, Slamon et al., 1989). The role of ErbB3 in causing cancer is underpinned by the formation of a heterodimer with dysregulated ErbB partners (such as ErbB1 and ErbB2), mainly ErbB2. The role of ErbB4 in driving oncogenesis is complex as it has multiple isomeric forms with varied functionalities: some are tumourigenic and others tumour suppressive (Sundvall et al., 2008). ErbB signalling was demonstrated to inhibit apoptosis in keratinocytes, epithelial cells and diverse tumour cells (Stoll et al., 1998, Tikhomirov and Carpenter, 2004, Yamaoka et al., 2008). In this chapter, I have shown for the first time that the ErbB family kinases are important regulators in a myeloid cell survival pathway.

Neutrophils undergo spontaneous apoptosis *in vitro* over a timecourse of approximately 30 hours (Lee et al., 1993, Savill et al., 1989b). Both Erbstatin analog and Tyrphostin AG825 accelerated spontaneous neutrophil apoptosis *in vitro*, which possibly suggests that ErbB activation is required for constitutive neutrophil survival responses, although the mechanism for this is not clear. A number of polypeptide ligands bind with the members of ErbB family kinases (except ErbB2 which lacks a ligand binding site). These ligands are synthesised as transmembrane polypeptide precursors which later on are converted to soluble forms and active N-terminal ectodomains by proteolytic

cleavage (Massague and Pandiella, 1993, Singh and Harris, 2005). Although most ligands are secreted or shed from the surface membrane, some ligands may act in a juxtacrine manner by which they are neither secreted nor shed, but rather may bind with the receptor kinases by direct contact (Massague and Pandiella, 1993, Singh and Harris, 2005). All known high-affinity ErbB ligands have an EGF -like domain that is the main determinant for the binding or activation of the kinases. The known ligands for ErbB1 are EGF, HP-EGF (Hairpin-binding EGF), TGF (Transforming Growth Factor) α, EPG (Epigen), AR (Amphiregulin), EPR (Epiregulin) and BTC (Betacellulin). Among these HP-EGF, EPR and BTC also bind with ErbB4, which also binds other classes of ligands called neuregulins (NRGs). All four types of neuregulins such as NRG1, NRG2, NRG3, and NRG4 can bind with ErbB4; however, NRG1 and NRG2 also bind with ErbB3. Though both Erbstatin analog and Tyrphostin AG825 induced neutrophil apoptosis, ErbB ligands such as EGF (ligand for ErbB1) and NRG-1 (ligand for ErbB3/4) did not enhance neutrophil survival responses, perhaps suggesting that ErbB survival signalling in neutrophils is mainly mediated by ErbB2 possibly in the form of a constitutively active homodimer. Nonetheless, the activation of ErbB signalling may be mediated by ligands that may be present in culture media, which may cause the receptors to be activated, or via the production and release of ligands in an autocrine manner, or can possibly be activated by ligands within the surface membrane from nearby cells in a juxtracrine manner, for example the interaction between TGFα (an ErbB1 ligand) and ErbB1 (Anklesaria et al., 1990). Constitutive ErbB activation also can possibly be explained by a transactivation mechanism via heterologous signalling pathways (such as signals from G-protein coupled receptors, GPCRs) that may feed into and regulate ErbB pathway. ErbB2 homodimers can be constitutively active since there is no ligand binding site and therefore ErbB2 is tightly regulated by its intrinsic autoinhibitory activity within the cytoplasmic domain (Fan et al., 2008). This autoinhibitory activity is mainly mediated by a loop formed by interaction between the αC helix and β4 plated sheet within the cytoplasmic kinase domain (Fan et al., 2008). The inherent autoinhibition mechanism

within the cytoplasmic kinase domain can prevent continual ErbB2 activation (Fan et al., 2008), and may possibly explain the constitutive apoptosis of neutrophils *in vitro.*

The IC_{50} value of Erbstatin analog for ErbB1 is 0.75 µM (0.15 µg/ml) (Umezawa et al., 1990), and the IC_{50} value of Tyrphostin AG825 for ErbB2 is 0.35 µM (Osherov et al., 1993). The concentration of Erbstatin analog and Tyrphostin AG825 used in this study to get the biological effect were high (40 µM and 50 µM, respectively), which would fail 10 µM cut off used for the screening process executed in chapter 4. Since both of these inhibitors are ATP-competitive and compete with ATP for binding to ATP-binding sites of the kinases (Osherov et al., 1993, Umezawa and Imoto, 1991), high concentration of inhibitors may be required to compete with high concentrations of intracellular ATP (millimolar concentration range) (Beis and Newsholme, 1975), which are concomitant with the literature where supramaximal concentrations of Tyrphostin AG825 were used to get biological effects (Levitzki and Gazit, 1995, Murillo et al., 2001, Osherov et al., 1993). Moreover, Tyrphostins such as AG825, have low aqueous solubility (Levitzki and Gazit, 1995) which may limit the actual solubilised inhibitors in cell culture media and which subsequently reduces the bioavailability of the inhibitor to cells. These considerations may explain the requirement of high concentration of inhibitors to get cellular effects on apoptosis.

Since EGF and neuregulin-1 did not show any effects on apoptosis and high concentration of the inhibitors (Erbstatin analog or Tyrphostin AG825) had effects, other potential off-target effects of ErbB inhibitors within neutrophils cannot be ruled out. None of the possible off-target effects were not studied, which is one of a major limitation in this study. However, the specificity of ErbBs inhibitors at the concentrations used in this study could be addressed if I could show the reduction of phosphorylation of ErbBs in presence of these inhibitors. The possible off-target effects of Erbstatin analog or Tyrphostin AG825 could be determined by using an antibody microarray analysis or phospho-proteomics approach if these inhibitors can affect on the reduction of phosphorylation of other kinases involved in unrelated pathways.

Since the inhibitor compounds screen identified ErbB2 inhibitors and our group have shown by antibody microarray analysis ErbB2 is phosphorylated in response to N^6-MB-cAMP (**Appendix 7.7**), ErbB2 may be the most effective target when considering potential drug targets for inflammatory diseases. However, further studies are warranted to confirm the role of ErbB2 in neutrophil survival and inflammation. As genetic manipulation of *in vitro* derived human neutrophils is intractable, genetic deletion of ErbBs is not possible. However, it may be possible to address this by generating ErbB2 specific zebrafish or murine transgenic models (ErbB2 functional mutant), but this was beyond the scope in my PhD because of time limitations.

The ErbB family RTKs transduce signals through diverse signalling pathways. The output of ErbB signalling networks not only depends on ErbB receptor activation but also input from other signals initiated by hormones, cytokines, growth factors and stress inducers (Carpenter, 1999). This transregulatory activity is mainly mediated by protein kinases that phosphorylate and therefore activate the kinase activity of ErbB family members (Carpenter, 1999). ErbB activation transduces signals through a number of well described cell survival/apoptosis cascades such as PI3K, AKT, p38 MAPK and Bcl-2/-x etc (Yarden and Sliwkowski, 2001). Importantly, PI3K pathway activation has been noted to induce cellular survival signals and also allows cells to resist the effects of apoptosis stimuli in many cell types (Cantley, 2002, Yao and Cooper, 1995), including neutrophils (Cowburn et al., 2002). PI3K kinase dependent survival responses signal via activation of AKT (also called protein kinase B) which is a key and immediate downstream event in PI3K pathway activation (Dudek et al., 1997, Kauffmann-Zeh et al., 1997, Khwaja et al., 1997). Taken together, therefore ErbB inhibition is expected to prevent neutrophil survival, which is consistent with this study as both Tyrphostin AG825 and Erbstatin analog promoted neutrophil apoptosis *in vitro*.

In addition to increased neutrophil constitutive apoptosis, both Erbstatin analog and Tyrphostin AG825 reversed the effects of multiple prosurvival stimuli that signal via diverse signalling pathways. These results suggest the ErbB family kinases may be

considered as potential targets when designing therapy for inflammatory diseases in the lung since diverse prosurvival mediators are present in the lung during inflammation. GMCSF plays an important role in airway inflammation by inhibiting neutrophil apoptosis and is found at elevated levels in BAL of patients with chronic inflammatory disease (Klein et al., 2000, Wicks and Roberts, 2016, Yasui et al., 2002). The GMCSF-mediated neutrophil survival response is generated by activation of PI3K/AKT pathway, leading to stabilization of anti-apoptotic Bcl-2 family member, Mcl-1(Derouet et al., 2004, Klein et al., 2000). The lung is exposed to LPS as a result of microbial infections that are often associated with COPD exacerbations. LPS prolongs neutrophil survival via a TLR4 dependent mechanism which subsequently activates MAPK, PI3K and NF-kB pathway (Sabroe et al., 2002, Sabroe et al., 2003, Ward et al., 1999, Ward et al., 2005, Ward et al., 2004). N6-MB-cAMP is a cAMP analog and generates a neutrophil survival response via a PKA-dependent mechanism (Krakstad et al., 2004, Vaughan et al., 2007, Martin et al., 2001), which is distinct to LPS or GMCSF-mediated survival signals. In spite of these distinct pathways, ErbB inhibitors prevented the survival response, implying that ErbB pathways are dominant prosurvival pathways and inhibition of this kinase family can overcome the effects of other signalling pathways occurring in parallel. Reversal of LPS-mediated survival responses may be mediated by PI3K/AKT pathway as AKT can phosphorylate IKKα, therefore generating NF-kB activity (Bai et al., 2009), which is a key signalling player in response to LPS (Sabroe et al., 2003, Ward et al., 2005). AKT can also phosphorylate cAMP Responsive Element Binding protein (CREB) which is a key factor in inducing cAMP-mediated cellular responses (Du and Montminy, 1998), and thus inhibition of ErbB may alter CREB via modulating the activity of the PI3K/AKT pathway. Furthermore, a quantitative protein-protein interaction network study reports that although each ErbB kinase can potentially bind with numerous cytosolic proteins involved in various signalling pathways, PI3K binds with ErbB kinases with high specificity (Jones et al., 2006). Therefore, the PI3K/AKT pathway may be a key downstream control point that feeds into multiple distinct pathways stimulated by these

stimuli. To examine this in the context of neutrophil survival, I have shown that Tyrphostin AG825 prevented GMCSF-induced AKT phosphorylation, suggesting that Tyrphostin AG825 reversed GMCSF-mediated survival via the alteration of PI3K activation. Mcl-1 plays an important role in neutrophil survival and Mcl-1 levels have been demonstrated to correlate with neutrophil survival (Michels et al., 2005, Moulding et al., 1998). Moreover, the level of both constitutive and GMCSF-induced Mcl-1 was reduced following Tyrphostin AG825 treatment. Since Tyrphostin AG825 modulated early pro-survival signalling by inhibiting PI3K/AKT activation which is a key downstream pathway of the ErbB signalling, and also prevented Mcl-1 levels at 8 hours, the ErbB-mediated survival response, therefore, may partly be conveyed through the PI3K/AKT pathway. Therefore, these results suggest that inhibition of ErbB mechanistically alters neutrophil survival pathways. In addition to the PK3/AKT pathways, other potential pathways such as p38 MAPK and Bcl-2/-x may also play roles as downstream cascades, as ErbB RTKs are known to regulate heterologous signalling. It has been reported that Tyrphostin AG825 promotes apoptosis via p38 MAPK-dependent pathways in prostate cancer cells (Murillo et al., 2001), implying possible engagement of p38 MAPK pathway downstream of ErbB signalling. However, further work is needed to establish this.

Neutrophils were also obtained from patients with COPD to test the effects of Erbstatin analog and Tyrphostin AG825, since COPD circulatory neutrophils demonstrate altered functionality (Burnett et al., 1987, Fietta et al., 1988, Milara et al., 2012, Noguera et al., 2001, Prieto et al., 2001, Sapey et al., 2011, Woolhouse et al., 2005), including reduced constitutive apoptosis during exacerbations (Pletz et al., 2004). Circulatory neutrophils from COPD patients were equally sensitive to Erbstatin analog or Tyrphostin AG825 induced apoptosis, suggesting that ErbB inhibitors may have potential in inflammation resolution via acceleration of neutrophil apoptosis in patients with systemic inflammation.

In this study alongside COPD neutrophils, ErbB inhibitors were tested in neutrophils from age-matched healthy control subjects, as the literature suggests that

innate immune functions of neutrophils such as phagocytosis (Butcher et al., 2001, Wenisch et al., 2000), ROS generation (Fulop et al., 2004), degranulation (McLaughlin et al., 1986), NET formation (Hazeldine et al., 2014), microbicidal activity (Simell et al., 2011, Wenisch et al., 2000), are altered in elderly subjects. Furthermore, it has been noted that that neutrophil apoptosis in the presence of survival factors (such as GMCSF, LPS) was shown to be higher in elderly subjects (although spontaneous apoptosis was unchanged) (Fulop et al., 1997, Tortorella et al., 1999, Tortorella et al., 1998, Tortorella et al., 2006), suggesting that the regulation of neutrophil apoptosis is also altered in elderly subjects. Both Erbstatin analog and Tyrphostin AG825 promoted apoptosis in neutrophils from elderly subjects.

Since the inhibition of ErbBs promoted neutrophil apoptosis and reversed neutrophil prosurvival responses in COPD patients, ErbB inhibition may serve as a potential therapeutic strategy for this disease. Since COPD patients studied in this cohort had frequent exacerbations and no differences in neutrophil apoptosis were observed between COPD patients and age-matched healthy control subjects, the patients who are colonised with pathogens may be immune-compromised as a result of a possible neutropenic effect of ErbB inhibition. However, normalising the intensity of the ErbB inhibition while retaining the immune functions of neutrophils may overcome the issue.

As opposed to other forms of cell death such as necrosis and necroptosis, apoptosis is an anti-inflammatory cell death mechanism and therefore it was important to confirm that ErbB inhibition engaged apoptosis. Using flow cytometry based detection via annexin-V and Topro-3 staining, the effects of Erbstatin analog and Tyrphostin AG825 were found to enhance apoptosis rather than necrosis as these inhibitors did not alter Topro-3 positive events. Furthermore, ErbB inhibitor driven apoptosis was completely abrogated by the pan-caspase inhibitor, Q-VD-OPh, which supported the conclusion that effects of ErbB inhibitors are via apoptosis and not an alternative death process. The engagement of apoptosis by these inhibitors was further validated by the loss of Mcl-1. Notably, the acceleration of neutrophil apoptosis has been reported to

reduce inflammation in multiple disease *in vivo* models (Chello et al., 2007, El Kebir and Filep, 2013b, Heasman et al., 2003, Ren et al., 2008, Rossi et al., 2006). These observations emphasise the importance of studying ErbB inhibitors *in vivo* models of inflammation. Therefore, the effects of Tyrphostin AG825 were studied in two *in vivo* models: zebrafish tail injury model and LPS-induced acute lung inflammation murine model.

Though zebrafish have long been used as a model organism for studying vertebrate development and haematopoiesis, it is now also considered as an excellent model for studying immunology, including inflammation biology. Studying neutrophil mediated innate immunity using the zebrafish larvae model is advantageous as larvae are transparent, allowing real-time monitoring of fluorescently labelled proteins in cellular processes *in vivo*. Furthermore, zebrafish neutrophils appear to be functional approximately 48 hours after fertilisation (Lieschke et al., 2001), while adaptive immunity does not develop for a further 4-6 weeks (Trede et al., 2004). In this study, a transgenic mpx:GFP zebrafish line expressing GFP under the neutrophil-specific myeloperoxidase (mpx) promoter was adopted to visualise the neutrophilic response *in vivo*. Tyrphostin AG825 was found to reduce the number of neutrophils at the site of inflammation while the total number of neutrophils was unaltered. This is perhaps because inflammatory neutrophils are more sensitive to Tyrphostin AG825 or that compensatory mechanisms may take place to replenish circulatory neutrophils. The reduced number of neutrophils at the site of inflammation could possibly be attributed by reduced neutrophil chemotaxis or increased apoptosis or by enhanced reverse migration (Ellett et al., 2015, Mathias et al., 2006). Whether the reduced number of neutrophils at the inflammatory site is a specific reflection of increased apoptosis was not established due to time constrictions and lack of sensitive apoptosis detection approaches in fish. However, it may be possible to address this in the future by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining, as described in literature (Elks et al., 2011, Robertson et al., 2014). Further to this, it may also be possible to determine whether this decrease is a result of

reverse migration phenotype (using photoconvertible neutrophil-specific transgenic lines, *Tg(mpx:Gal4/UAS:Kaede)* (Elks et al., 2011, Yoo and Huttenlocher, 2011) or of a recruitment phenotype (Robertson et al., 2014) as described previously. The reduced number of neutrophils at the inflammation site, following Tyrphostin AG825 treatment, suggests that ErbB2 plays roles in inflammation and may be a potential therapeutic target in resolving inflammation. Since only the ErbB2-selective inhibitor Tyrphostin AG825 was tested in the fish tail injury model, other inhibitors selective for other members of ErbB RTKs needs to be tested to determine their role in inflammation *in vivo*. Furthermore, it is also important to validate this finding by generating or obtaining ErbB transgenic fish, or by using anti-sense oligonucleotide morpholinos (Moulton, 2007). For elucidation of the role of ErbB kinases in diverse phenotypes in fish, a number of studies has been performed using either genetic manipulation of ErbB RTKs by using morpholinos (Budi et al., 2008, Honjo et al., 2008, Rojas-Munoz et al., 2009), suggesting the feasibility of adopting these techniques to validate the role of ErbB RTKs in inflammation.

In order to determine a role for ErbB RTKs in resolving inflammation in a mammalian model, I moved into a murine model of acute lung inflammation. This model also allowed us to directly measure neutrophil apoptosis and clearance *in vivo*, which is technically challenging in zebrafish. In contrast to other agents for inducing lung injury, LPS was used in this study since LPS is a well-established inducer of inflammation, is easy to administer (in nebulised form), and also provides a study in a context relevant to bacterial infection, which is commonly associated with COPD exacerbations. In acute LPS-models, LPS generates a mixed inflammatory response, including increased neutrophils (Faffe et al., 2000, Lefort et al., 2001, Menezes et al., 2005, Thompson et al., 2013), in addition to other inflammatory cells and soluble mediators (Lee et al., 2007, Vernooy et al., 2002) in the lung. A number of studies have shown that the peak neutrophilic response in the lung was observed at 24 hours following LPS administration, followed by a gradual reduction of the response in a time dependent manner (Faffe et al., 2000, Lefort et al., 2001, Menezes et al., 2005), and neutrophil apoptosis is well

documented to cause this decline (Menezes et al., 2005, Brass et al., 2008). In this murine model, Tyrphostin AG825 was administered intraperitoneally to inhibit ErbB *in vivo* at a dose of 20 mg/kg, which is consistent with a previous study (Kedrin et al., 2009). In this study, neutrophil apoptosis and efferocytosis were studied at 48 hours which is one of the time points where neutrophil apoptosis contributes to the decline of neutrophil numbers in the lung and is also consistent with the previously published literature (Thompson et al., 2013). In this study, Tyrphostin AG825 was found to increase neutrophil apoptosis and macrophage-driven efferocytosis in BAL at 48 hours post treatment. However, the proportion or the total number of neutrophils was unaltered, possibly because of simultaneous recruitment of neutrophils at this time point and however, later time point may show a reduction of neutrophil and total cell number. The ability of Tyrphostin AG825 to enhance neutrophil apoptosis at a single time point does not mean that its effect contributes to inflammation resolution, however further studies with multiple time points may be required to see the effect of Tyrphostin AG825 on neutrophilic inflammation resolution in the lung. It is well-established that an efficient inflammation resolution process in acute lung inflammation is correlated with increased neutrophil apoptosis (El Kebir et al., 2012). Therefore, the increased neutrophil apoptosis with Tyrphostin AG825 may contribute to inflammation resolution in the lung, and this could be addressed if pan-caspase inhibitor can prevent the Tyrphostin AG825-induced reduction of infiltrated of neutrophils and other inflammatory cells in the lung in the murine model.

Since Tyrphostin AG825 induced apoptosis *in vivo,* the results are consistent with the ability of this inhibitor to promote apoptosis *in vitro* and since Tyrphostin modulated the PI3K/AKT pathway, resulting in Mcl-1 modulation *in vitro*, it can be speculated that Tyrphostin AG825 driven apoptosis *in vivo* may be conveyed by the PI3K/AKT and Mcl-1 dependent mechanisms. However, further studies are required to address this and involvement of other potential pathways.

As a proof of principle, Tyrphostin AG825 was tested and found to promote neutrophil apoptosis in the murine model system, suggesting ErbB2 as a potential component of neutrophilic inflammation and a therapeutic target. However, the role of other members of ErbB family should be investigated to see if they also have the same role in neutrophil survival and inflammation *in vivo*. A transgenic mice with a dominant negative allele of ErbB2 (carboxyl-terminal truncated) has been developed to study the role of ErbB2 in the development of the mammary gland (Jones and Stern, 1999), suggesting the feasibility of studying the ErbB2 kinase using a transgenic murine model and may help to investing the role of this kinase in inflammation *in vivo*.

This is the first study showing the role of ErbB kinases in myeloid cells. Though small molecule inhibitors of ErbB function are in clinical use as anti-cancer agents, they might have the potential for repurposing as drugs in treating inflammatory diseases.

Conclusions:

Inhibition of ErbB1 and ErbB2 with selective small molecule inhibitors, Erbstatin analog and Tyrphostin AG825 respectively, accelerated spontaneous neutrophil apoptosis and reversed the effects of a number of neutrophil prosurvival factors in COPD and healthy control subjects. Using the zebrafish tailfin injury model, Tyrphostin AG825 was shown to reduce the number of neutrophils at the site of inflammation. Tyrphostin AG825 was also shown to enhance neutrophil apoptosis and alveolar macrophage efferocytosis in an acute lung inflammation model. This is the first study revealing the role of the ErbB family of RTKs in regulating neutrophil survival.

Thus the findings in this chapter identified protein kinase dependent pathways involved in inflammation resolution with potential application as a novel therapeutic strategy for chronic inflammatory diseases.

Chapter 6. General Discussion

Host immune cells, including neutrophils, have the ability to limit infection, inflammation and tissue damage in a highly regulated manner as part of a homeostatic response to infection and injury. Neutrophil phagocytosis and apoptosis play important roles in this context, and these processes are known to be dysregulated in acute and chronic inflammatory disease (Donnelly and Barnes, 2012, Hoenderdos and Condliffe, 2013, Zhang et al., 2012). Identifying the molecular targets involved in these processes and therefore the mechanisms underpinning the dysregulation is a foremost need in order to develop therapies for inflammatory diseases. In this study, I have attempted to understand cellular processes in chronic inflammation, particularly emphasising on COPD, and also aimed to identify therapeutically targetable novel molecular targets regulating neutrophil apoptosis pathway(s).

6.1. Key findings in the study

Neutrophils are key immune effector cells, providing defence against bacterial and fungal infections (Kaufmann, 2008, Kolaczkowska and Kubes, 2013). However, they have the potential to contribute deleterious effects on host tissues, as implicated in acute and chronic inflammatory diseases such as COPD, where neutrophilic inflammation drives pathogenesis and disease progression (Brown et al., 2009, Haslett, 1999, Pletz et al., 2004, Zhang et al., 2012). Under these inflammatory settings, neutrophil functions such as phagocytosis and apoptosis are abnormally regulated under the influence of multiple microenvironmental factors.

Neutrophils are professional phagocytes, and yet bacteria persist in the lungs of patients with chronic inflammation despite the tissue neutrophilia, suggesting that the phagocytic capacity of neutrophils may be compromised in this disease (Stockley, 2002). In this study, I found that COPD neutrophils have a modest phagocytic defect based on a reduction of the phagocytic index, although both the % of neutrophils containing the

bacteria and the mean bacteria per neutrophil did not alter. Furthermore, the roles of a number of key inflammatory factors (IFN-γ, mROS, and hypoxia) in the modulation of neutrophil phagocytosis were studied; however, the phagocytic capacity was not regulated in the presence of these inflammatory mediators.

I found a moderate phagocytosis defect in COPD based on a single parameter, however, the effect may not be the effect of COPD rather may reflect the effect of ageing of the patients, since healthy subjects were much younger individuals. Therefore, the phagocytosis defect identified in this thesis is inconclusive. Although circulatory neutrophil apoptosis did not reduce in COPD compared to age-matched healthy subjects, as discussed before in this thesis, existing studies with a considerably higher number of subjects show that circulatory neutrophil apoptosis was reported to reduce in COPD (Pletz et al., 2004, Schmidt-Ioanas et al., 2006, Zhang et al., 2012) ; however, no change was also reported (Noguera et al., 2004). Even with this controversy whether circulatory neutrophil apoptosis in COPD is suppressed or not, delayed neutrophil apoptosis at sites of inflammation has been implicated in the pathophysiology of COPD and other inflammatory diseases, which prevents the removal of these potentially toxic cells and as a consequence, the resolution of inflammation (Hoenderdos and Condliffe, 2013, Whyte et al., 1993, Zhang et al., 2012). Driving neutrophil apoptosis is a valuable therapeutic strategy for inflammatory diseases, as evidenced by a number of studies where this approach promotes inflammation resolution experimentally (Burgon et al., 2014, Chello et al., 2007, Heasman et al., 2003, Ren et al., 2008, Rossi et al., 2006). In the past few decades, although many advances in the understanding of neutrophil apoptosis have been documented, more research is required to identify drugable molecular targets regulating this pathway. In light of this, an unbiased screening process on neutrophil apoptosis was adopted and identified a number of kinases which may have potential roles in regulating neutrophil apoptosis. Among the kinases identified, I focussed on the role of the family of ErbB RTKs, since the kinases of the family were a common targets and was inhibited by some of the most specific compounds. I have

shown that inhibition of these kinases *in vitro* not only promoted neutrophil apoptosis but also overrode a number of neutrophil prosurvival stimuli and mechanistically alters neutrophil survival pathway as shown by reducing the AKT activation and the levels of Mcl-1. Since neutrophil function is influenced by a number of cellular and soluble factors *in vivo*, I explored the role of ErbBs in models of zebrafish and murine inflammation. The ErbB2 inhibitor promoted inflammation resolution and neutrophil apoptosis in these models. Therefore, this study suggests a possibility of targeting the ErbB RTKs for the development of therapeutics for inflammatory diseases.

6.2. Limitations

Although some limitations of this study have already been discussed in chapters 3-5, a number of general limitations require discussion.

There are number of limitations in the experimental model systems in neutrophil research. For example, the phenotypic or functional responses of neutrophils are affected by the surrounding environment during *in vitro* experiments which can induce variability in the results between experiments rendering a difficulty while working with neutrophils. Donor variability is another concern, often making data interpretation difficult and meaning sizeable data sets are required. Furthermore, since neutrophils are terminally differentiated cells and have a short lifespan, these cells are not suitable for genetic manipulation experiments and therefore all of the findings in this thesis are derived from pharmacological tools.

An important limitation is that I was unable to study the expression of ErbB RTKs in neutrophils. The Kinex microarray data suggested activation (and therefore expression) of ErbB2 in response to N^6-MB-cAMP as evidenced by the detection of a phosphorylated protein product. An undergraduate student in the Prince group has since demonstrated

the expression of ErbB2 in human neutrophils, which is regulated by the survival stimuli GMCSF and cAMP.

Further limitation in this study is that the ability of Tyrphostin AG825 was tested in a single time point in the *in vivo* murine model system; therefore, the reduced neutrophil numbers observed in Tyrphostin AG825 treatment group did not provide any information regarding the ability of the inhibitor in inflammation resolution. Furthermore, Tyrphostin AG825 was not administered after establishment of inflammation in the lung, rather administered at the same time of LPS nebulisation and only tested only in an acute inflammation setting. Thus this model system does not mimic the scenario of chronic inflammation, for instance COPD, and/or acute COPD exacerbation; however this could be addressed by adopting COPD models as discussed detail below in section 6.4.

6.3. Therapeutic implications

Although current therapies for inflammatory diseases, for example bronchodilators and steroids used in COPD, can reduce the symptoms, they do not have potential to reverse the pathological processes and do not specifically target neutrophilic inflammation. Since over 400 human diseases are linked to abnormal activities of kinases (Melnikova and Golden, 2004), kinase inhibitors are increasingly being exploited therapeutically. In the context of inflammatory diseases, a number of studies highlighted that kinases are important regulators of neutrophil apoptosis (Burgon et al., 2014, Juss et al., 2012, Rossi et al., 2006, Wang et al., 2003, Webb et al., 2000). My study suggests ErbB RTKs as potential drug targets for inflammatory diseases. As discussed, aberrant activation of ErbB RTKs has been reported to contribute to the development of multiple carcinomas (Roskoski, 2004), and small molecules inhibitors (such as lapatinib, erlotinib, and gefitinib) and mAb (trastuzumab, panitumumab, and cetuximab) are in clinical use as therapeutics in cancer medicine (Ather et al., 2013, Singh et al., 2016). Furthermore, a number of potential candidates such as dacomitinib are in clinical evaluation (Ather et

al., 2013, Singh et al., 2016). Since my study showed that the ErbB inhibition promotes neutrophils apoptosis and reduces neutrophil burden at the sites of inflammation, this study has opened up a possibility of repurposing the ErbB inhibitor drugs for achieving therapeutic benefits in chronic inflammatory diseases, including COPD. Repurposing the ErbB inhibitor based drug for treating chronic inflammatory disease would be beneficial not only because of their safety to use has already been established but also this approach is a least-resource intensive. ErbB inhibitors may possibly be able to be considered for drugs in the case of COPD exacerbations; however, further work is warranted to establish if these inhibitors have the ability to reduce inflammation in experimentally established COPD and its exacerbation models. Cancer patients treated with ErbB inhibitor drugs (such as Trastuzumab) have been reported to show neutropenia as one of the side effects (Ghani et al., 2014, Inada-Inoue et al., 2014), and this neutropenia may be explained by the enhanced neutrophil apoptosis as a result of inhibition of ErbB RTKs, which is consistent with our finding in this study. If this is the case, while considering ErbB inhibitors as drugs for inflammatory diseases, the patients treated with these inhibitors may be immunocompromised as a result of neutropenia. This is a potential side effect and particularly a major concern if the patients are chronically colonised with pathogens. However, targeted drug delivery approach to the inflammatory sites, for instance, by delivering the ErbB inhibitors in nebulised form to the lung for COPD, may be fruitful for targeting the inflammatory neutrophils more readily than systemic neutrophils.

6.4. Further questions to address

Although this thesis describes a role for ErbB RTKs in neutrophil survival and inflammation, further work is required to address the detailed mechanisms underpinning this. Although this thesis suggests that the ErbB-driven neutrophil survival involves the PI3K/AKT pathway and Mcl-1, other key survival signalling pathways (such as p38 MAPK,

ERK MAPK and Bcl-2/-x) were not explored and required further study. Furthermore, the roles of these signalling pathways are needed to focus on in parallel *in vivo* contexts.

Although the inhibition of ErbBs promoted neutrophil apoptosis *in vivo* and *in vitro*, and reduced inflammation *in vivo*; however, further studies are required to address how this inhibition affects other key neutrophil functions such as phagocytosis and chemotaxis. Furthermore, the effects of ErbB inhibition have not been studied in other cell populations (e.g. macrophages, epithelial cells etc.) residing in the inflammatory environments. These studies are essential as the ErbB inhibitors can be considered as potential therapeutics for inflammatory diseases if they have the ability to reduce inflammation while retaining the key functions of neutrophils and other immune and epithelial cells.

Although this study shows the ErbB inhibition reduces neutrophil burden in acute inflammation *in vivo*, it is needed to establish further whether the inhibition of ErbBs has the ability to improve clinical outcomes or disease severity scores in established chronic inflammatory disease models, which would require further studies to confirm the therapeutic efficacy of ErbB inhibitors. In particular, I would adopt a murine model of COPD by chronically intranasal administration of LPS/NE once a week for a month to generate an established inflammation as previously described (Sajjan et al., 2009), following which a further acute inflammation (to mimic acute exacerbation) will be provoked with LPS in the same route followed by an administration of Tyrphostin AG825 and analysing ability of this inhibitor in inflammation resolution and improving clinical outcomes. In addition to murine models of lung inflammation, the finding in this thesis could be strengthened by testing the ErbB inhibitors in additional mammalian models of inflammation, such as zymosan-induced murine models of peritonitis, bleomycin-induced lung injury and passively induced arthritis etc (Jonsson et al., 2005, Kolaczkowska et al., 2010, Rossi et al., 2006).

6.5. Conclusions

This study identified a novel role for ErbB family kinases in neutrophil survival pathway and inflammation. To my knowledge, this is the first study demonstrating a role of ErbB family kinases in myeloid cell function. The ability of ErbB inhibitors in the acceleration of neutrophil apoptosis and inflammation resolution, suggests a repurposing potential of existing ErbB inhibitors currently in clinical use in cancer medicine for treating chronic inflammatory diseases.

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Chapter 7. Appendices

Appendix 7.1. Hypotonic lysis buffer (500 µl)

Reagents	Stock conc.	Working conc.	Required volume
Protease Inhibitor cocktail (Calibiochem)		1:100	5 µl
Phenylmethanesulfonyl fluoride (PMSF) (AppliChemPanreac)	100mM	1mM	5 µl
Diisopropylfluorophosphate (DFP) (Sigma-Aldrich)	100mM	1mM	5 µl
Sodium Fluoride (NaF) (Sigma-Aldrich)	1 M	50 mM	25 µl
Sodium orthovanadate (Sigma-Aldrich)	1 M	10 mM	5 µl
dH_2O			455 µl
Total			=500 µl

Appendix 7.2. 2X SDS buffer (10ml)

Reagents	Stock conc.	Working conc.	Required volume
1,4-Dithio-DL-threitol (DTT) (Tocris Bioscience)	1M	0.1 M	1 ml
20 % SDS solution	20 %	4 %	2 ml
Glycerol (Acros Organics)	100 %	20 %	2 ml
Tris-HCl (pH 6.8) (Bio-Rad Laboratories)	0.5M	0.0625 M	1250 µl
Bromophenol Blue (Sigma-Aldrich)	0.2 %	0.004 %	200 µl
dH_2O			3.55µl
Total			=10 ml

Appendix 7.3. Reagents used in western blotting

20% APS (freshly made)

Reagents	Amounts
Ammonium persulfate (APS) (Sigma-Aldrich)	0.2g
dH_2O	1ml

10 X Running Buffer

Reagents	**Amounts**
Glycine (Fisher Scientific)	190 g
Tris Base (Fisher Scientific)	30.3 g
20% SDS solution (Fisher Scientific)	50 ml
dH_2O	1000 ml

1 X Transfer Buffer

Reagents	**Amounts**
Tris Base (Fisher Scientific)	2.9g
Glycine (Fisher Scientific)	1.45g
20% SDS solution	925µl
Methanol (VWR Chemicals)	100 ml
dH_2O	500ml

10 X TBS

Reagents	**Amounts**
Tris-HCl 1M pH 8.0 (Bio-Rad Laboratories)	100ml
NaCl (Fisher Scientific)	97.3g
dH_2O	1000ml

1 X TBS

Reagents	**Amounts**
10 X TBS	100 ml
dH_2O	1000 ml

10 X TBS-Tween

Reagents	**Amounts**
Tris-HCl 1M pH 8.0 (Bio-Rad Laboratories)	100 ml
NaCl (Fisher Scientific)	97.3 g
Tween-20 (Fisher Scientific)	5 ml
dH_2O	1000 ml

1 X TBS-Tween

Reagents	**Amounts**
10 10 10X TBS-Tween	M 100 ml
dH_2O	to 1000ml

Blocking Solution (5% skimmed milk solution)

Reagents	**Amounts**
Skim Milk Powder (Sigma-Aldrich)	5 g
1 X TBS	100 ml

Appendix 7.4. Primary and secondary antibodies used in Western blotting

Primary antibodies	Molecular size (~KDa)	Dilution	Secondary antibodies	Dilution
Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060 (Cell Signalling Technology)	60	1:800	Polyclonal Goat Anti-Rabbit Immunoglobins-HRP (Dako)	1:1000
Akt Rabbit mAb #9272 (Cell Signalling Technology)	60	1:1000	Polyclonal Goat Anti-Rabbit Immunoglobins-HRP (Dako)	1:1000
Mcl-1 (S-19) Rabbit polyclonal IgG (Santa Cruz Biotechnology)	40	1:300	Polyclonal Goat Anti-Rabbit Immunoglobins-HRP (Dako)	1:1000
Mouse Anti-Human p38 alpha (MAP Kinase) Monoclonal IgG1 (1 mg/ml) Catalog No. SMC-152 (StressMarq Biosciences Inc.).	38	1:2000	Polyclonal Goat Anti-Mouse Immunoglobins-HRP (Dako)	1:1000

Appendix 7.5. E3 Medium

Reagents	Amount (for 10X E3)
NaCl (5 mM)	2.92 g
KCl (0.17 mM)	1.3 g
CaCl2 (0.33 mM)	4.4 g
MgSO4 (0.33 mM)	8.1 g

1X E3 was made by diluting in distilled water with 0.05% methylene blue as anti-fungal agent (optional).

Appendix 7.6. The PKIS inhibitor compounds that enhanced ≥2-fold apoptosis in the primary screen.

Compounds	Apoptosis Fold change (of DMSO control)	Compounds	Apoptosis Fold change (of DMSO control)
GW589933X	8.63	GW576924A	2.69
GW305074X	8.57	GW297361X	2.64
GW441756X	8.52	GSK317354A	2.64
GW305178X	7.26	GW445017X	2.60
GSK579289A	6.37	GW567808A	2.56
GSK943949A	4.65	GW831091X	2.53
GSK1751853A	4.46	SB-686709-A	2.47
SB-278538	4.21	GW832467X	2.44
GW631581B	4.08	SB-376719	2.41
GW513184X	4.06	GSK237700A	2.40
SB-245392	3.99	GW440139A	2.39
GW830365A	3.89	GW820759X	2.39
GW795493X	3.87	GW406108X	2.37
SB-751148	3.85	GSK204925A	2.31
GSK1000163A	3.79	GSK994854A	2.29
GSK978744A	3.78	GW827106X	2.27
GW442130X	3.71	GW683109X	2.27
GW627834A	3.68	SB-736290	2.25
GSK1326255A	3.64	GW814408X	2.17
SB-242719	3.59	GW829055X	2.16
GW831090X	3.56	GW580496A	2.11
GW569293E	3.36	GSK614526A	2.10
GW680908A	3.34	GW781673X	2.09

GSK237701A	3.33	GW616030X	2.09
GSK1173862A	3.23	GW770249A	2.08
GW296115X	3.04	GW301784X	2.07
GW574782A	3.04	GW694590A	2.05
GW784307A	3.04	GW607049C	2.04
GW796920X	2.75	GW407323A	2.02
GW284372X	2.72	SB-278539	2.02
GW406731X	2.72	GW459057A	2.01

Appendix 7.7. Kinexus antibody microarray analysis

Ultrapure neutrophils were treated with N^6-MB-cAMP [100 µM] for 5, 30 and 60 minutes followed by immediately lysing the neutrophils. Lysates from 4 donors were then pooled and subjected to antibody microarray using 812 antibodies, including phospho-antibodies, specifically to measure the activation of target proteins in response to N^6-MB-cAMP.

Target Protein Name	**Phospho Site (Human)**	**Z-ratio (5, 0)**
PAK2	Pan-specific	7.84
PKCa/b2	T638/T641	6.42
IRAK4	Pan-specific	5.85
PDK1	S241	5.00
MEK3 (MAP2K3)	Pan-specific	2.71
FAS	Pan-specific	2.29
RSK1/3	T359+S363/T356+S360	2.14
TPIPb	Pan-specific	2.10
Src	Pan-specific	2.09
S6Ka (p70 S6Ka) + S6Ka (p85 S6Ka)	T412	1.87
Pyk2	Y579	1.84

IKKa	Pan-specific	1.83
GSK3a + GSK3b	Pan-specific	1.82
Msk1	S376	1.77
MEK3 (MAP2K3)	S218	1.76
MEK2 (MAP2K2)	Pan-specific	1.75
AMPKa1/2	T183	1.73
Smac/DIABLO	Pan-specific	1.73
PRK1 (PKN1)	Pan-specific	1.68
Progesterone Receptor	S294	1.68
PLCg1	Y783	1.68
IGF1Rb/IRb	Y1161/Y1185	1.67
Grp94	Pan-specific	1.66
GSK3a + GSK3b	S21	1.65
ErbB2 (HER2)	Pan-specific	1.64
PDI	Pan-specific	1.62
MSH2	Pan-specific	1.60
Shc1	Y349+Y350	1.60
Calnexin	Pan-specific	1.56
Target Protein Name	**Phospho Site (Human)**	**Z-ratio (30, 0)**
PDK1	S241	5.69
ZAP70/Syk	Y319/Y352	4.85
JIK (TAO3)	Pan-specific	4.44
PAK2	Pan-specific	4.16
IKKb	Pan-specific	3.78
p38a MAPK	T180+Y182	3.21
PLCg1	Y783	3.16
Plk2	Pan-specific	3.06
JNK1/2/3	Pan-specific	2.93

Plk1	Pan-specific	2.93
CDK1 (CDC2)	Pan-specific	2.80
MEK1 (MAP2K1)	T386	2.70
ROS	Pan-specific	2.58
FKHRL1	T32	2.58
PKG1a	Pan-specific	2.54
GSK3a + GSK3b	S21	2.54
Huntingtin	S421	2.29
BLNK	Y84	2.25
MEK1 (MAP2K1)	Pan-specific	2.14
PKBb (Akt2)	Pan-specific	2.08
Jun	S73	1.99
Rb	S608	1.99
ErbB2 (HER2)	Y1248	1.94
Btk	Y223	1.92
ROKa (ROCK2)	Pan-specific	1.91
Crystallin aB	Pan-specific	1.86
Bad	S99	1.81
Plk3	Pan-specific	1.74
DNAPK	Pan-specific	1.73
AMPKa1/2	T183	1.70
Synapsin 1	S605	1.69
PKBa (Akt1)	S473	1.64
p38g MAPK (Erk6)	Pan-specific	1.64
STAT1a + STAT1b	Pan-specific	1.60
ErbB2 (HER2)	Pan-specific	1.59
Src	Pan-specific	1.57
ERP72	Pan-specific	1.51

Target Protein Name	Phospho Site (Human)	Z-ratio (60, 0)
PAK2	Pan-specific	6.52
PDK1	S241	4.79
JIK (TAO3)	Pan-specific	4.64
I1PP2A	Pan-specific	4.09
Plk1	Pan-specific	3.34
ROS	Pan-specific	2.84
Plk2	Pan-specific	2.78
IKKb	Pan-specific	2.75
CDK1 (CDC2)	Pan-specific	2.74
PKCa/b2	T638/T641	2.73
ZAP70/Syk	Y319/Y352	2.72
S6Kb (p70 S6Kb)	Pan-specific	2.61
JNK1/2/3	Pan-specific	2.56
p38a MAPK	T180+Y182	2.32
PKG1a	Pan-specific	2.19
S6Ka (p70 S6Ka) + S6Ka (p85 S6Ka)	T412	2.05
Chk2	Pan-specific	2.02
Rb	S608	1.96
CK1g2	Pan-specific	1.90
Plk3	Pan-specific	1.88
PKBb (Akt2)	Pan-specific	1.85
PP2A B' (B56)	Pan-specific	1.83
PKCg	T514	1.79
STAT1a + STAT1b	Pan-specific	1.75
Src	Pan-specific	1.72
RSK4	Pan-specific	1.71

p38g MAPK (Erk6)	Pan-specific	1.65
PKCb1	Pan-specific	1.63
ROKa (ROCK2)	Pan-specific	1.61
PP4C	Pan-specific	1.59
ErbB2 (HER2)	Y1248	1.53
IkBa	Pan-specific	1.52