THE UNIVERSITY *of* EDINBURGH

CYCLIN-DEPENDENT KINASE INHIBITOR DRUGS DRIVE NEUTROPHIL GRANULOCYTE APOPTOSIS BY TRANSCRIPTIONAL INHIBITION OF THE KEY SURVIVAL PROTEIN MCL-1

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DECLARATION

I HEREBY DECLARE THAT THE DATA PUBLISHED IN THIS THESIS ARE THE RESULT OF MY OWN WORK CARRIED OUT UNDER THE SUPERVISION OF PROF. ADRIANO G ROSSI, DR RODGER DUFFIN AND PROF. CHRISTOPHER HASLETT AT THE UNIVERSITY OF EDINBURGH. THIS THESIS HAS BEEN COMPLETED ENTIRELY BY MYSELF AND HAS NOT PREVIOUSLY BEEN SUBMITTED FOR ANY OTHER DEGREE OR QUALIFICATION.

ANDREW LEITCH

ABSTRACT

The normal physiological response to bacterial infection or wounding with threat of infection, termed inflammation, has been shown to be dysregulated in certain human diseases including (but not limited to): idiopathic pulmonary fibrosis, acute lung injury, arthritis and glomerulonephritis. The earliest arriving and most abundant cell responding to an inflammatory stimulus is the neutrophil granulocyte. It has been shown that under inflammatory conditions neutrophil granulocytes have extended longevity, enhanced responsiveness and upregulated activation parameters. In the setting of non-infective, or prolonged, ineffectually-cleared infective disease where resolution of inflammation does not occur then neutrophil granulocytes may cause tissue damage which is mediated by excessive, misdirected exocytosis of toxic granule contents or by spillage of the same products from necrotic or netotic cell carcasses that have lost membrane integrity. A key process in the resolution of inflammation is the induction of apoptosis in recruited neutrophils following a successful response to an inflammatory stimulus. Cellular signalling from apoptotic cells and from professional phagocytes that have ingested apoptotic cells has been shown to favour resolution of inflammation and restoration of tissue homeostasis. Additionally, the removal of key inflammatory cells in a highly regulated, non-phlogistic fashion robustly assists the resolution process.

Cyclin-dependent kinase (CDK) inhibitor drugs are being developed as anti-cancer agents as it is hypothesized that they should interfere with the enhanced cell-cycling ability (increased proliferative capacity and extended longevity) which is such a key feature of cancer cell biology. The CDKs that drive the cell cycle are CDKs 1, 2, 4 and 6 and consequently agents were designed to have enhanced specificity for these targets. CDK inhibitor drugs target the ATP-binding domain of CDKs and as a result usually have activity against more than one CDK. The CDK inhibitor drug, R-roscovitine which targets CDKs 2, 5, 7 and 9 was shown to

promote neutrophil apoptosis and consequently resolution of inflammation. This thesis aims to investigate the mechanism by which apoptosis is induced in neutrophil granulocytes by CDK inhibitor drugs.

The first experimental chapter of this thesis explores in detail the time-course and active concentration range of CDK inhibitor drugs in comparison to known promoters and inhibitors of neutrophil apoptosis. It then dissects the apoptotic machinery which is responsible for the effects of CDK inhibitor drugs before investigating their capacity to promote apoptosis even in the presence of survival mediators relevant to the context of inflammatory disease. Flow-cytometry, light and confocal microscopy as well as western blotting for caspases, mitochondrial dissipation assay, fluorometric caspase assay and the detection of DNA laddering demonstrate that CDK inhibitor drugs promote classical neutrophil apoptosis by the intrinsic pathway and show similar kinetics of apoptosis induction to drugs that inhibit transcription.

The second experimental chapter investigates the key neutrophil survival protein and bcl-2 homologue Mcl-1. By flow cytometry, western blotting and RT-PCR it is demonstrated that Mcl-1 is down-regulated at the level of transcription and that this occurs even in the presence of inflammatory mediators that would normally promote neutrophil survival. Additionally, it is shown that pro-apoptotic bcl-2 homologues are affected to a lesser degree suggesting an imbalance of bcl-2 proteins is caused by effects at a transcriptional level mediated by CDK inhibitor drugs.

The third experimental chapter identifies CDKs and their binding partner cyclins in neutrophil granulocytes and investigates the impact of CDK inhibitor drugs on CDK protein levels and cellular distribution by differential lysis and western blotting as well as by confocal microscopy. The key transcriptional enzyme RNA polymerase II is also identified and the effect of CDK inhibitor drugs on

phosphorylation of this enzyme is documented. Western blotting and confocal microscopy demonstrate the presence of key CDKs 2, 5, 7, 9 and cyclin binding partners of CDKs 7 and 9. It is shown that the phosphorylation of RNA polymerase II mediated by CDKs 7 and 9 is inhibited by CDK inhibitor drugs. This suggests that a key mechanism by which neutrophil apoptosis is induced by CDK inhibitor drugs is the inhibition of transcription of key proteins and suggests that neutrophils require survival proteins for functional longevity.

The fourth experimental chapter addresses the production and use of HIV-tat dominant negative CDK 7 and 9 proteins to knockdown CDKs 7 and 9 in neutrophil granulocytes *in vitro* to provide a molecular biology surrogate for the pharmacological data already presented. The cloning, production, purification and use of HIV-tat dominant negative CDK proteins are described.

The final chapter describes the use of a more specific pharmacological inhibitor of CDKs 7 and 9, DRB, in the mouse bleomycin lung injury model. Resolution of inflammation by a compound specifically targeting CDKs 7 and 9 is described.

This thesis identifies CDKs 7 and 9 as key targets of CDK inhibitor drugs in neutrophilic inflammation. It shows these drugs acting at the level of transcription to drive neutrophil apoptosis by exploiting the unique dependency of neutrophils on the short-lived survival protein Mcl-1. In so doing the presence of functional and essential transcriptional machinery is identified in neutrophils and the transcriptional profile of resting, stimulated and inhibited neutrophils is delineated. These findings suggest novel approaches to the pharmacological promotion of resolution of inflammation and indicate key new targets for rational drug design. In future, it will be important to further characterize the effects of CDK inhibitor drugs on other cell-types including epithelial cells, fibroblasts and mononuclear cells. This information should prove important to the continued investigation of

CDK inhibitor drugs in resolution of inflammation and also to the ongoing experimental trial of these drugs in idiopathic pulmonary fibrosis.

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Abbreviations

18FDG	18-fluorodeoxyglucose
AA	arachidonic acid
Akt-1	v-akt murine thymoma viral oncogene homolog 1
ALI	acute lung injury
AMP	ampicillin
APCs	antigen-presenting cells
ARDS	adult respiratory distress syndrome
ATP	adenosine triphosphate
Bak	Bcl-2-antagonist/killer-1
BAL	bronchoalveolar lavage fluid
Bax	Bcl-2 associated X protein
BCL	B-cell lymphoma
Bid	BH3 interacting domain death agonist
Bim	Bcl-2 like 11 (apoptosis facilitator)
BMP-4	bone morphogenic protein-4
BP	binding protein
CAD	caspase activated DNAse
CD	cluster differentiation
CDK	cyclin-dependent kinase
CEBP	CCAAT-enhancer-binding protein
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator gene
CFU	colony forming unit
c-myc	v-myc myelocytomatosis viral oncogene homolog (avian)
COPD	chronic obstructive pulmonary disease
COX	cyclo-oxygenase
CREB	cAMP responsive element binding protein
CSF	colony stimulating factor
CT	computed tomography
CTD	C-terminal domain
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C motif) receptor
DAD	diffuse alveolar damage
DFF	DNA fragmentation factor
dibutyryl	db
DISC	death inducing signal
DMSO	dimethyl sulfoxide
dn	dominant negative
DNA	deoxyribonucleic acid
DRB	5,6-dichloro-1-β-D-ribofuranosyl-1H-benzimidazole
ECP	eosinophil cationic protein
ECP	endothelial cell
EDN	eosinophil-derived neurotoxin
EGF	epidermal growth factor
EPO	eosinophil peroxidase
ERK	extracellular signal regulated kinase
Ets	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)

FADD	Fas receptor associated death domain
FasL	fas ligand
FL	Flt-3 ligand
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FMT	fluoroscence molecular tomography
FP6	IL-6/IL-6 receptor fusion protein
GATA-3	GATA binding protein-3
GEMM	granulocyte-erythroid-monocyte-megakaryocyte
GFP	green fluorescent protein
GM	granulocyte macrophage
GO	gene ontology
GPCRs	g-protein coupled receptors
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
h	hour(s)
HDAC	histone deacetylase
HIV	human immunodeficiency virus
HL-60	Human promyelocytic leukemia cell-line
HRP	horseradish peroxidase
i.p.	intraperitoneal
i.t.	intra-tracheal
i.v.	intravenous
ICAD	inhibitor of CAD
ICU	intensive care unit
Ig	immunoglobulin
IKK	I-kappa-B-kinase
IL	interleukin
IPF	idiopathic pulmonary fibrosis
JAK	janus kinase
KAN	kanamycin
LEF-1	lymphoid enhancer binding factor-1
LPS	lipopolysaccharide
LT	leukotriene
MAPK	mitogen associated protein kinase
MAT1	menage a trois-1
MBP	major basic protein
Mcl-1	myeloid cell leukaemia-1
MCP-1	monocyte chemotactic protein-1
min	minute(s)
MIP-2	macrophage inflammatory protein
ML-1	myeloblastic cell-line
MMP	matrix metalloproteinase
MODS	multi-organ dysfunction syndrome
MOMP	mitochondrial outer membrane premeabilisation
MPO	myeloperoxidase
MRI	magnetic resonance imaging
mRNA	messenger RNA
MULE	HECT, UBA and WWE domain containing 1

NETS	neutrophil extracellular traps
NFAT	Nuclear factor of activated T-cells
NF-kB	nuclear factor-kappa-B
NSAIDs	non-steroid anti-inflammatory drugs
NZBxNZW	New Zealand Black/New Zealand White mouse cross
OD	optical density
PAF	platelet activating factor
PCR	polymerase chain reaction
PEST	domain rich in proline, glutamic acid, serine and threonine
PET	positron emission tomography
PFA	paraformaldehyde
PG	prostaglandin
PI	propidium iodide
PI3K	phosphoinositol-3-kinase
PL	phospholipase
PM	particulate matter
PS	phosphatidylserine
P-TEFb	positive transcription elongation factor b
PU.1	transcription factor PU.1
Raf	v-raf-1 murine leukemia viral oncogene homolog 1
RAR	retinoic acid receptor
Rb	retinoblastoma protein
RNA	ribonucleic acid
RNA pol II	RNA polymerase II
ROS	reactive oxygen species
s	second(s)
s/c	subcutaneous
SAP	shrimp alkaline phosphatase
SCF	stem cell factor
siRNA	small interfering RNA
SRE	steroid responsive element
STAT	signal transducer and activator of transcription
TFIIH	transcription factor IIH complex
TGF	transforming growth factor
TH2	T-helper 2
TLRs	toll-like receptors
TNF-α	tumour necrosis factor-alpha
TNFR	TNF receptor
TPO	thrombopoietin
TRADD	Tumour necrosis factor receptor type-1 associated death domain
TRAF2	TNF receptor associated factor
TRAILR	TNF-related apoptosis-inducing ligand
TX	thromboxane
UV	ultraviolet
xl	extra long
YSPYSPS	Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7
zVAD-fmk	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone

1 Introduction

In this chapter I aim to review the published literature on inflammation, granulocytes (with specific regard to neutrophils and apoptosis), the survival protein myeloid cell leukaemia (Mcl)-1, cyclin dependent kinases (CDKs) and the bleomycin lung injury model. This information will provide a background to the major themes investigated in the thesis as a whole.

1.1 Lung Inflammation and Immunity

1.1.1 Structural immune defence in the lung

The respiratory mucosa is the largest body surface area to be exposed to and require defence from the external environment. It is not surprising, therefore, that a robust, vigorous and rapidly responsive immune defence system has evolved over millennia of exposure to diverse and increasingly sophisticated microbes. Additionally, air-borne irritant particulate matter (PM) such as allergens, biopersistent fibres such as asbestos and combustion-derived nanoparticles (e.g. diesel exhaust particles) must be dealt with in order to maintain a high level of function (Donaldson et al. 2005). The architecture of the respiratory system provides a first layer of defence against air-borne micro-organisms and PM effectively blocking the lower airways from anything greater than 5μm in diameter (Donaldson et al. 2005). Inhaled air is warmed and filtered through the nasal hair and nasopharyngeal passages before negotiating the larynx (which ordinarily prevents any intrusion from matter destined for the gastrointestinal system and allows phonation) down the large tubular trachea and into the upper bronchi. The initially large bronchial airways repeatedly bifurcate until they reach an extremely narrow gauge and form alveoli at which point gas exchange with the capillary network that lines the alveolar wall becomes possible. Pseudo-stratified columnar ciliated epithelium predominates in the trachea and bronchi forming the muco-ciliary escalator. Any intruding micro-organism or misplaced particulate matter slipping out of the air-stream

in the upper airways is trapped in the muco-ciliary escalator and forced up and out by the regular beating motion of the respiratory cilia (Leitch et al. 2008).

1.1.2 Innate immune defence in the lung

Should these initial defences be breached then an intruder must be adapted to survive an uninviting but non-specific chemical milieu including lysozyme, endogenous antimicrobial agents, adverse pH, Immunoglobulin (Ig) A and surfactants (Seaton 2000). Should this fail then a more goal-directed system is brought on-line. The respiratory mucosa has developed a sensitive and specific recognition strategy which allows it to identify microbe specific molecular patterns such as lipopolysaccharide (LPS), lipotechoic acid, formylated peptides, flagellin and non-methylated DNA. Once detected these galvanise resident alveolar macrophages and initiate a variety of pro-inflammatory pathways that instigate the classical elements of inflammation (calor (heat), rubor (redness), dolor (pain), tumor (swelling) and loss of function as described by Celsus and Virchow) driven by increased vascular permeability leading to a proteinaceous infiltrate and leucocyte recruitment. The Toll-like receptors (TLRs) mediate most of this form of recognition and though they each have a specific role TLRs can function in unison to expand their powers of recognition (Sabroe et al. 2007b). Resident alveolar macrophages deal with the majority of insults that trigger this alert system but if they are overwhelmed recruited leucocytes including granulocytes lend assistance.

The invading microbes are rendered highly recognisable to recruited granulocytes by comprehensive opsonisation mediated by both complement-dependent and independent means. They are then ingested (phagocytosed) by neutrophils or, if they should prove resistant to this because of size or learned subversion are subjected to a chemical onslaught (exocytosis) as neutrophils forcibly externalize toxic granule substances such as lactoferrin and myeloperoxidase (MPO) as well as reactive oxygen species (ROS) (Rossi et al. 2007;Serhan et al. 2007). A subset of neutrophils (and other leucocytes potentially (von Kockritz-Blickwede et al. 2008)) employ web-like neutrophil extra-

cellular traps (NETs) to ensnare and kill resistant organisms but must themselves die in the process (Brinkmann et al. 2004). In beneficial neutrophil-dominant inflammation the organisms or foreign particles are detected and phagocytosed by neutrophils which then undergo an organized, non-provocative programmed-cell death (apoptosis) which promotes their own recognition and removal by macrophages or dendritic cells. At this point the interface between the innate and adaptive immune systems occurs as macrophages, which migrate to the lympho-reticular system following ingestion of apoptotic neutrophils, act as antigen presenting cells (APCs) allowing the lymphocyte population to complete the resolution and remembrance process. The next time that particular organism is encountered a pre-prepared, specific response should be available to ensure it has less opportunity to make an impact (Nathan 2006).

The lungs are the arena for another type of granulocyte-driven inflammatory response. Eosinophils are present in larger numbers within the lungs of asthma sufferers and are recruited in greater numbers in response to sensitizing allergens such as pollen, house dust mite and animal dander. This response has no obvious beneficial effects and it is still unclear why it should occur. The eosinophil is a useful and active defender against parasitic infection and has an armament specific to that end. It has been noted that since parasitic infections have been largely eradicated in Western society the incidence of this abnormal eosinophil response (termed allergy or atopy) has increased and it seems that eosinophilic inflammation occurs almost as an outlet for redundancy (Leitch et al. 2008;Seaton 2000).

1.1.3 Acute and chronic inflammation and disease

Inflammation is part of the beneficial anti-microbial, immune defence system that has been honed and conserved by evolution over millions of years (Marchalonis et al. 2002). The system has become increasingly sophisticated because of the breadth of micro-organisms the human body has encountered and because of the mechanisms these organisms have evolved to enable evasion of its front-line defences. Ideally, following

prompt detection of a micro-organism by immune mechanisms, an inflammatory reaction should contain and destroy the organism before it multiplies, spreads, becomes established or causes harm. Streptococcal lobar pneumonia can be paradigmatic of a beneficial inflammatory response as despite a massive, neutrophil-dominant inflammatory reaction, resolution of inflammation occurs (and did so in the majority of cases in the pre-antibiotic era) with no pathological damage (Haslett 1999). Self-regulation and limitation are the key, final components of the response as the system must actively drive resolution of inflammation to restore tissue homeostasis (Bystrom et al. 2008;Nathan 2006;Rajakariar et al. 2008). There is now abundant evidence that inflammatory cell (especially neutrophil) programmed cell death (or apoptosis, initially described by Kerr et al 1972) followed by non-phlogistic clearance of apoptotic cells by phagocytes such as macrophages plays a key role in ensuring efficient (and in the majority complete) resolution of inflammation.

Inflammatory diseases arise for a number of reasons; some of which are not fully understood. If the inflammatory system cannot destroy organisms or foreign particles (such as asbestos fibre and silica) it malfunctions, initiating excessive or prolonged inflammatory reactions which are likely to lead to irreversible fibrosis and scarring. Alternatively, reactions may be sited where none are apparently needed or self-antigens become recognised as foreign. In these settings chronic inflammatory and autoimmune disease may supervene. The outcome of excessive or persistent (chronic) inflammation is tissue damage caused by the secretion or loss of histotoxic products from over-activated, under-regulated or frankly necrotic inflammatory cells (Fadok et al. 1998;Rossi et al. 2007;Voll et al. 1997). The spectrum of inflammatory disease includes all the major organ systems and, increasingly, we are recognising a role for inflammation in other disease processes such as cancer (Coussens et al. 2002;Hussain et al. 2007), neurological disorders (Gilgun-Sherki et al. 2006), atheroma (Hansson et al. 2006) and menstrual dysfunction (Critchley et al. 2001). There are many similarities between inflammatory diseases but also important differences which may occur even within the same organ system. For example, in lung–based disease, inflammation in

subsets of asthma is eosinophil-dominant while in chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) it is arguably neutrophil-dominant (Barnes 2007;Kinder et al. 2008;Nair et al. 2009;Walsh 2000) (Figure 1).

1.1.4 Resolution of inflammation

The resolution phase of inflammation has emerged as a key target for the development of novel anti-inflammatory therapies. The key elements required for resolution of inflammation to proceed towards tissue homeostasis have been defined as: removal of the inciting stimulus, controlled death (usually by apoptosis) of inflammatory leucocytes and removal of dead cells and debris by phagocytic cells (Figure 1) (Rossi et al. 2008;Serhan et al. 2007). This has led to the redesign of some traditional models of inflammation and approaches to their investigation (Navarro-Xavier et al. 2009;Renshaw et al. 2006). Importantly the first fruits of this movement are beginning to appear with the likely translation of the compound RX-10045 (based on the lipid mediator resolvin) which has multiple pro-resolution properties to clinical usage for the treatment of inflammation related dry-eye (www.resolvyx.com).

Many inflammatory diseases are treated with glucocorticosteroid drugs either independently or in combination regimens. Glucocorticosteroids work through a variety of effects at the molecular level including enhancement of anti-inflammatory and repression of pro-inflammatory gene transcription (Newton 2000). They are very successful at treating eosinophil-dominant inflammatory disease such as asthma while they have had more modest (possibly arguable) success with neutrophil-dominant disease. It is now known that glucocorticoids promote eosinophil apoptosis but actually prolong neutrophil survival (Heasman et al. 2003;Sivertson et al. 2007;Ward et al. 1999). The latter effect is, presumably, partially compensated by glucocorticoid-mediated, enhanced macrophage phagocytosis of apoptotic neutrophils (Heasman et al. 2003). The development of non-steroidal anti-inflammatory drugs (NSAIDs) and subsequently Cyclo-oygenase (COX)-2 specific NSAIDs demonstrate further the

difficulties associated with combating a well-established, integral host response such as inflammation. NSAIDs work by inhibiting the COX enzymes 1 and 2. COX-1 is constitutively expressed in all tissues while COX-2 is largely an inducible enzyme subject to various regulatory factors. These enzymes convert arachidonic acid (AA) to prostaglandin (PG)-H_2 which can be converted by isomerise enzymes to: PGE_2, $PGF_{2\alpha}$, PGD_2, prostacyclin (PGI_2), and thromboxane (TX)A_2. PGE_2 is responsible for some of the classic features of inflammation. Traditional NSAIDs inhibited COX-1 and 2 resulting in side-effects related to sites where COX-1 has constitutive housekeeping duties (such as the gut leading to peptic ulcer disease), COX-2 specific inhibitors were an attempt to avoid these side-effects. Unfortunately, this specific inhibition appeared to confer a greater risk of vascular thrombotic events (such as heart attack and stroke) thought to be related to suppression of PGI_2, an atheroprotective agent, but not COX-1 derived TXA_2, a pro-aggregatory and vasoconstrictor mediator. However, recent work has suggested that it may be possible to exploit the anti-inflammatory and pro-resolution effects of PGD_2 metabolites (Usually responsible for negative feedback inhibition of the inflammatory response) which inhibit nuclear-factor-kappa-B (NF-κB) and have been shown to resolve inflammation in animal models (Lawrence et al. 2002;Rossi et al. 2007;Ward et al. 1999). Another significant barrier to the successful design of novel anti-inflammatory agents has been the complex, multi-layered structural redundancy that characterises the inflammatory system. Where one cytokine pathway is knocked down, another may compensate and the whole is maintained. Only the removal of a load-bearing structure will allow collapse and return to homeostasis.

Recent anti-inflammatory approaches have utilised ‘biological therapies’ in the targeting of specific cytokines. Anti-tumour necrosis factor (TNF)-α therapy is the stand-out success of this genre and there is now considerable evidence that TNF-α is the sought after load-bearing structure in inflammatory disease though other agents are showing promise (Feldmann et al. 2002;Williams et al. 2007). It is clear that with such redundancy in the system there will always be room for new pharmacological agents that have novel targets within the inflammatory system.

Figure 1

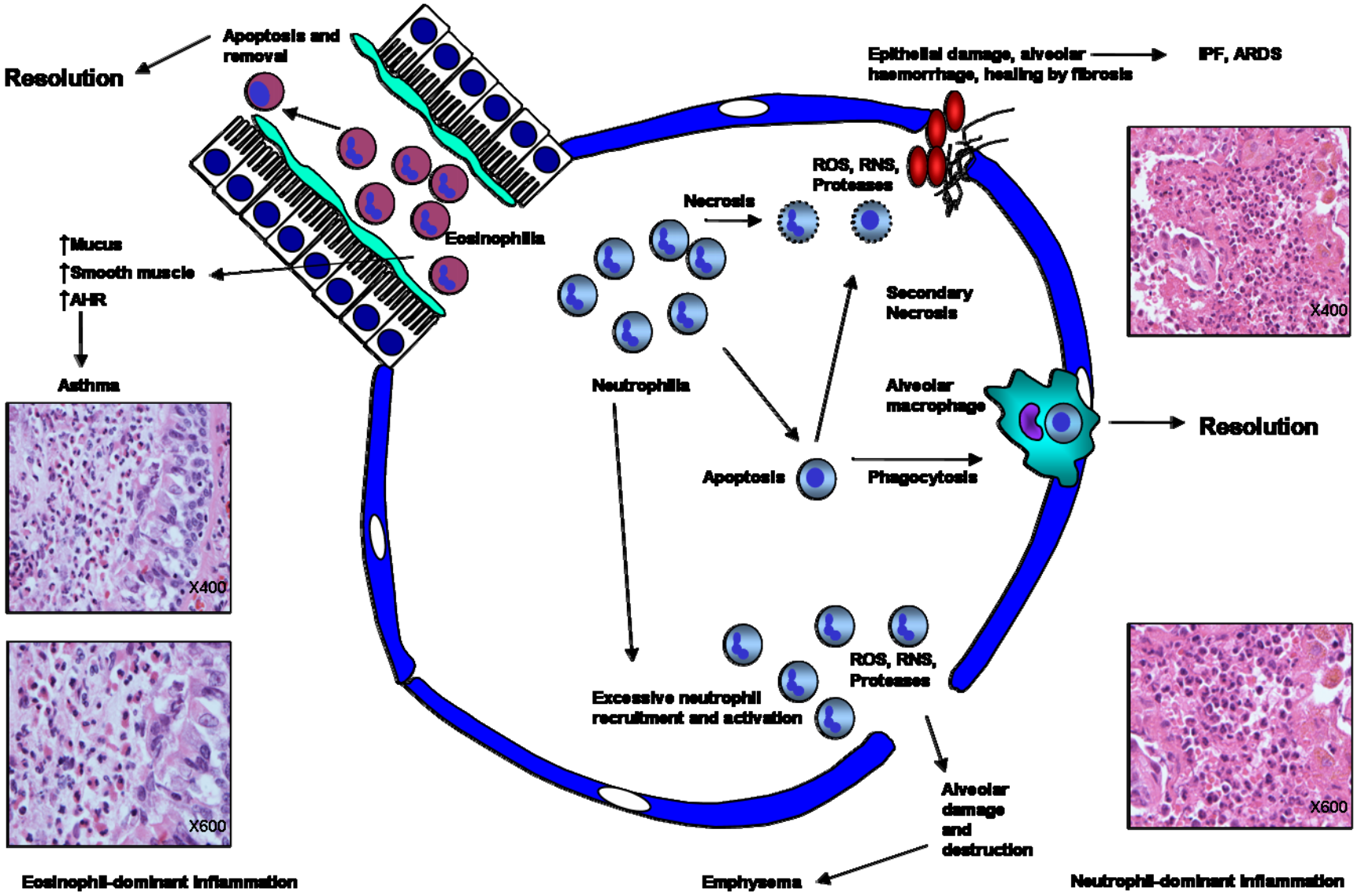

Resolution
Apoptosis and removal
Epithelial damage, alveolar haemorrhage, healing by fibrosis
IPF, ARDS
ROS, RNS, Proteases
Necrosis
Eosinophilia
↑Mucus
↑Smooth muscle
↑AHR
Asthma
Secondary Necrosis
Neutrophilia
Alveolar macrophage
Resolution
Apoptosis
Phagocytosis
X400
X400
X600
ROS, RNS, Proteases
Excessive neutrophil recruitment and activation
Alveolar damage and destruction
X600
Eosinophil-dominant inflammation
Emphysema
Neutrophil-dominant inflammation

Figure 1: Diagram showing both resolution and failure of resolution of eosinophil and neutrophil dominant inflammatory processes. Neutrophilia at the respiratory mucosa is resolved by apoptosis of infiltrating neutrophils and phagocytic clearance by macrophages. It fails to resolve where neutrophils are in great excess or are not efficiently cleared and undergo secondary necrosis following apoptosis. This leads to alveolar damage and destruction followed by fibrotic healing. Histology on the right hand side of diagram shows neutrophil-dominant inflammation. Eosinophil-dominant inflammation is resolved by the same mechanisms and fails to resolve for the same reasons. The effects of eosinophil-dominant inflammation in the asthmatic airway are depicted and an example of the histology demonstrated on the left-hand side of the diagram. Histology was kindly provided by Dr William Wallace (Pathology Dept. Edinburgh Royal Infirmary).

A critical step in the development of pro-resolution of inflammation agents is demonstrating efficacy in animal models. Our own work, focused on the use of cyclin-dependent kinase inhibitors to drive granulocyte apoptosis has utilised several murine models of inflammation, including passively-induced arthritis, bleomycin-induced lung injury and carrageenan-elicited acute pleurisy. Other strategies that have been shown to resolve inflammation in animal models include anti-recruitment agents like phosphoinositol-3-kinase (PI3K) inhibitors (Pinho et al. 2005;Pinho et al. 2007)/ anti-interleukin (IL)-5, multi-effect agents like resolvins/lipoxins (Serhan 2007) and designer antibody approaches against cytokines (anti-TNF-α, anti-IL-6, anti-IL-33, anti IL-17 (Feldmann et al. 2002)) and integrins (anti-cluster differentiation (CD)11b). Anti-cytokine therapies have of course translated to use in inflammatory disease but it is unlikely that a single therapy will be totally efficacious as there appears to be considerable inter-individual heterogeneity in the inflammatory response mounted to similar stimuli.

There have been recent exciting innovations in this area with the characterisation and utility of the zebrafish model and the increasingly rapid development of novel imaging modalities. It is also clear that researchers are beginning to think from a pro-resolution perspective in the development of models so that a more uniform set of read-outs of efficacy might be achieved. This is particularly evident in a thoughtful paper by Gilroy et al., which encourages the use of a mouse peritonitis model which they have extensively characterised (Navarro-Xavier et al. 2010).

The zebrafish model offers the advantages of a readily available *in vivo* system that can be monitored and modified at organism level and dissected at molecular level. The system has obvious experimental advantages but also has potential for use as a high-throughput pharmacological screening technique (Barros et al. 2008). Renshaw et al (Renshaw et al. 2006;Renshaw et al. 2007) have developed a transgenic zebrafish line that expresses green fluorescent protein (GFP) under the myeloperoxidase promoter allowing them to track all stages of the inflammatory process. In addition they have shown that they can alter the course of resolution by introducing the pan-caspase

inhibitor N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me)fluoromethyl ketone (zVAD-fmk) to prevent apoptosis and hence resolution. Conversely they have shown resolution of inflammation with the CDK inhibitor drug, R-roscovitine.

The benefit of inducing neutrophil apoptosis during pneumococcal meningitis was comprehensively shown by Koedel *et al* (Koedel et al. 2009). Neurological deficits caused by brain tissue damage are a common finding in survivors of severe meningitis and are linked to increased longevity of the neutrophil population recruited to the brain. Inducing neutrophil apoptosis with R-roscovitine in conjunction with antibiotic therapy with ceftriaxone reduced markers of neuronal damage in murine models of pneumococcal meningitis. This suggests a possible new strategy for the limitation of detrimental neurological side-effects of meningeal infection as well as opening up a more general approach to the management of severe infective disease where a causative role for neutrophils in disease pathology can be demonstrated. A dual strategy directed against both the causative organism and the inflammatory response generated toward that organism may be more efficacious than traditional microbe-directed treatment.

Imaging the process of inflammation and its resolution is key to understanding the pathophysiology involved and to the development of non-invasive screening tools for novel resolution agents. In early experiments using rabbits with experimental upper lobe pneumonia, Haslett *et al* (Jones et al. 1994) showed that 18-fluorodeoxyglucose (^{18}FDG) uptake detected by positron emission tomography (PET) could be used as a surrogate for neutrophil activity. PET is still at the forefront of inflammation imaging but novel techniques are emerging. Imaging modalities that have been shown to be of use in the assessment of inflammation include magnetic resonance imaging (MRI), computerised tomography (CT), fluorescence molecular tomography (FMT) and intravital fibre-optic confocal fluorescence microscopy. In particular MRI has been used to show inflammatory neutrophilic response *in vivo*, in stroke models by measuring MPO activity (Breckwoldt et al. 2008). This technique utilises an ‘MPO-sensing agent’ which gives an enhanced image compared to gadolinium alone. Additionally the same group

used fluorescence molecular tomography (FMT) and intravital confocal microscopy to monitor allergic airway inflammation in a mouse model (Cortez-Retamozo et al. 2008). In these experiments they used a matrix metalloproteinase (MMP)-sensing probe to exploit the unique enzymatic activities of eosinophils at the site of inflammation.

We have mainly focused on models of lung inflammation including: bleomycin (acute and chronic), LPS challenge, carrageenan pleurisy, ovalbumin peritonitis and ovalbumin airways challenge. These models are now extensively characterised in the literature and allow the assessment of classical inflammatory parameters including inflammatory cell subset and number, oedema, lavage protein level, cytokine levels and histological appearances. We have shown that zVAD-fmk also prevents resolution of these models and have demonstrated resolution of inflammation with CDK inhibitor compounds (Rossi et al. 2006). These pro-resolution agents have now been shown to selectively drive inflammatory cell apoptosis *in vitro* (Duffin et al. 2009;Leitch et al. 2010b), to resolve inflammation *in vivo* (measured by the parameters discussed above) (Rossi et al. 2006) and are about to be used in an experimental medicine trial in inflammatory lung disease. The key feature of these agents is their selectivity for inflammatory granulocytes and their paucity of effect on macrophage phagocytic clearance of apoptotic cells. Thus the benefits of driving apoptosis are retained whilst the deleterious potential creation of secondarily necrotic cells is avoided.

The apoptotic neutrophil and the process of cell death exert anti-inflammatory effects that have been shown to be of therapeutic value in inflammatory disease models. Our own work on the pharmacological manipulation of the apoptotic pathway (e.g., by using CDK inhibitor drugs) has resulted in improved disease scores in murine lung fibrosis, arthritis and pleurisy models (Rossi et al. 2006). The endogenous inhibitors of CDKs have also been a recent focus in macrophage inflammatory responses that have demonstrated a role for the endogenous CDK inhibitor p21 (waf1/CIP1) in attenuation of LPS-induced cytokine production by murine macrophages in sepsis models (Lloberas et al. 2009;Scatizzi et al. 2009). Recently, in a rather radical but nonetheless elegant

approach, Savill *et al* used the systemic addition of apoptotic neutrophils (made apoptotic *ex vivo*) as an anti-inflammatory therapy. In an LPS-driven mouse model of sepsis, enhanced survival of animals receiving apoptotic cells was observed, theoretically because apoptotic cells bound LPS and were subsequently cleared by macrophages (Ren et al. 2008). In this context, the benefit of apoptotic neutrophils was no doubt enhanced by their ability to confer a pro-resolution phenotype on macrophages thus curtailing any further inflammatory response.

1.2 Granulocytes, inflammation and inflammatory disease

Granulocytes, the collective name given to neutrophil, eosinophil and basophil leucocytes, play a prominent role in immune defence. Neutrophils, being the most abundant circulating granulocyte in human blood, play a key role in defence against bacterial, fungal and viral infections. Eosinophils account for less than 5% of the circulating granulocytes and confer resistance to parasitic invasion as well as being involved, with basophils (the third type of granulocyte and numerically scarce), in the allergic response. Granulocytes are the foot-soldiers of the inflammatory response and are dispatched in large numbers to overcome many challenges to the host organism. They are attracted by and follow a concentration gradient of chemotactic stimuli released by invading pathogens or tissues under challenge. They migrate from the circulation across post-capillary venule endothelial cells (or capillary endothelial cells in pulmonary inflammation (Downey et al. 1993)) and employ a formidable armamentarium to overcome their adversaries. Granulocytes are named for the numerous granules within their cytoplasm. Each of these granules contains a range of toxic products such as proteases, lysozyme and lactoferrin. Eosinophils have an armament more specific to the killing and digestion of parasites (including major basic protein, eosinophil cationic protein and eosinophil peroxidase) but, in asthma, respond to allergens or airway irritants in a concerted reaction which involves IgE, mast cells, lymphocytes, basophils and smooth muscle.

1.2.1 Granulocyte structure and function

The mature granulocytes, neutrophil, eosinophil and basophil are distinguished by differences in nuclear morphology, size, granule content and function. They will be discussed individually in the following passages.

Neutrophils

Neutrophils are 12-15μm in diameter and account for 70% of the circulating leucocyte population which corresponds to approximately 2-7.5x10^9 neutrophils. They survive in the circulation for approximately 7-10 hours but if compelled by inflammatory mediators or pharmacological agents can extend their lifespan up to 48h and it has recently been suggested that they may survive as long as 5 days *in vivo*. This population turnover requires efficient production by the bone marrow and prompt clearance by that same organ with help from the spleen and liver. Neutrophils are possessed of at least four different types of granule termed: primary (azurophilic), secondary (specific), gelatinase and secretory; each carries a specific arsenal of toxic chemicals that the neutrophil uses against non-host elements. The neutrophil is a key effector cell at the front-line of immune defence and is efficacious (and life-saving) in the majority of instances because of its versatility. Neutrophils prove effective phagocytes because on accomplishment of this task they are programmed to die a prompt, quiet and importantly, contained, apoptotic death. This limits the time available to internalized micro-organisms and prevents hijacking of cellular controls to enable subversion (as is thought to occur when *Mycobacterium tuberculosis* is internalized by macrophages (Sundaramurthy et al. 2007)). This timely death also signals larger-scale phagocytes such as macrophages, dendritic cells and epithelial cells to initiate a phagocytic response. If a neutrophil is unable to phagocytose an invader then it will disgorge granule contents into the surrounding environment causing tissue damage and amplifying the inflammatory response. This should not be construed as a purely detrimental response as it serves to block potential routes of entry for invading organisms as tissue damage causes capillary shut-down and collapses lymphatics, sends

a strong signal of imperilled defence and allows time for an alternative immune strategy to be adopted (Nathan 2006).

Neutrophils may also employ NETs to ensnare and kill resistant organisms. NET formation involves the extrusion of fine chromatin and granule coated tendrils that are microbicidal and fungicidal. This effort proves fatal to the neutrophil (Brinkmann et al. 2007). Finally, neutrophils are not just blunt effectors but also smooth operators responsible for negotiating the recruitment and education of other arms of the immune system. It is becoming appreciated that there is a false division between the innate and adaptive immune response systems and that a more realistic model involves a continual interplay between constituents of these systems. Neutrophils not only alert APCs and lymphocytes to danger but regulate their response to it and in return receive counter-regulation. Appropriate resolution of an inflammatory immune response is finely balanced (Nathan 2006;Serhan et al. 2007).

It is not surprising given the constant exposure of the vast respiratory mucosa to threat, the fine balance of the immune response and the pivotal inflammatory role played by neutrophils that neutrophil-dominant inflammation has been implicated in the pathophysiology of numerous inflammatory respiratory diseases including: pneumonia, COPD, IPF, cystic fibrosis (CF) and adult respiratory distress syndrome (ARDS)/acute lung injury (ALI).

Eosinophils

Eosinophils are approximately 12-17μm in size and under normal circumstances account for less than 5% of the circulating leucocyte population. They may survive for up to 12 hours in the circulation but have the ability to extend their longevity to over a week if required. Not only are they bigger than neutrophils but they also wear more flamboyant colours when stained by the Romanowsky method (methylene blue and eosin) which accounts for their name. Eosinophils may also be distinguished by their production of Charcot-Leyden crystals (manufactured from lysophospholipase an eosinophil-derived enzyme) which are often visible in their cytoplasm. Eosinophils, like neutrophils, are

supplied with numerous granules though their constituents differ including: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN). This array is certainly capable of tissue damage and probably evolved in order to combat helminthic infection. Increasingly, new roles for eosinophils are being identified and it seems likely that they have a role in combating viral infection as ECP and EDN have been shown to degrade single-stranded RNA viruses. They are said to play an early role in innate immunity by production of important cytokines such as IL-4 though this is probably only physiologically important in the gastrointestinal tract. They can also modulate adaptive immunity by specific activation of T-cells (Rothenberg et al. 2006).

Eosinophils are implicated in a different spectrum of disease from neutrophils, of which the most common variety is allergic/atopic disease which includes asthma, allergic rhinitis and eczema. In other countries parasitic infection is still common including schistosomiasis, dranunculoriasis, ascariasis, filariasis and hookworm. Eosinophil numbers may be increased in various cancers as well as in rare conditions such as Churg-Strauss, aspergillosis and eosinophilic pneumonia. In eosinophil-dominant disease it has been consistently demonstrated that eosinophil excess due to excessive recruitment, apoptosis avoidance and failed clearance has a correlation with disease pathology.

Basophils

Basophils are most often encountered in the literature as histamine-producing cells that appear to play second fiddle to mast cells in the IgE-driven response that is characteristic of allergic asthma. However they are a significant source of other cytokines including IL-4 and IL-13 and also contribute to the modulation of T-helper 2 (T_H2)-type inflammation by influencing the interactions of T-cells with dendritic cells (Lambrecht et al. 2009). The extent of their contribution to innate immune defence and their function and interplay in a wider immune context remain to be elucidated

1.2.2 Granulocyte genesis and differentiation

The life-cycle of the granulocyte and its differentiation in particular are under increasing scrutiny. Granulocytes, under the influence of well-characterised growth factors (IL-1, IL-6, granulocyte macrophage (GM)-colony stimulating factor (CSF) and IL-3) are differentiated from multi-potent stem cells in the bone marrow. They transition from stem cell through primitive progenitor cells called colony-forming unit (CFU) granulocyte-erythroid-monocyte-megakaryocyte (GEMM) and subsequently CFU-granulocyte macrophage progenitor cells (GM) into specific precursor lineages (Edwards 1994;Opferman 2007) (Figure 2). It is evident that a distinctive eosinophil/basophil progenitor cell exists termed CFU-Eo/Baso. The development of multipotential cells into CFU-Eo/Baso cells is controlled by SCF, IL-3, IL-4, GM-CSF, and eotaxin. The terminal differentiation into a mature eosinophil is driven by IL-5 and eotaxin (Gauvreau et al. 2009;Giembycz et al. 1999b). By contrast, neutrophil differentiation appears to be regulated by granulocyte (G)-CSF, IL-3, GM-CSF and lymphoid enhancer-binding factor 1 (LEF-1) (target genes *survivin, cyclin D1, CCAAT-enhancer-binding protein (CEBP)-α* and *v-myc myelocytomatosis viral oncogene homolog (avian)(c-myc)*) (Skokowa et al. 2006;Skokowa et al. 2007). This may vary under inflammatory conditions where there is knockout animal evidence that IL-1 and TNF-α can promote a reactive neutrophilia by indirect effects on myeloid progenitors in the absence of other differentiation factors (Ueda et al. 2009).

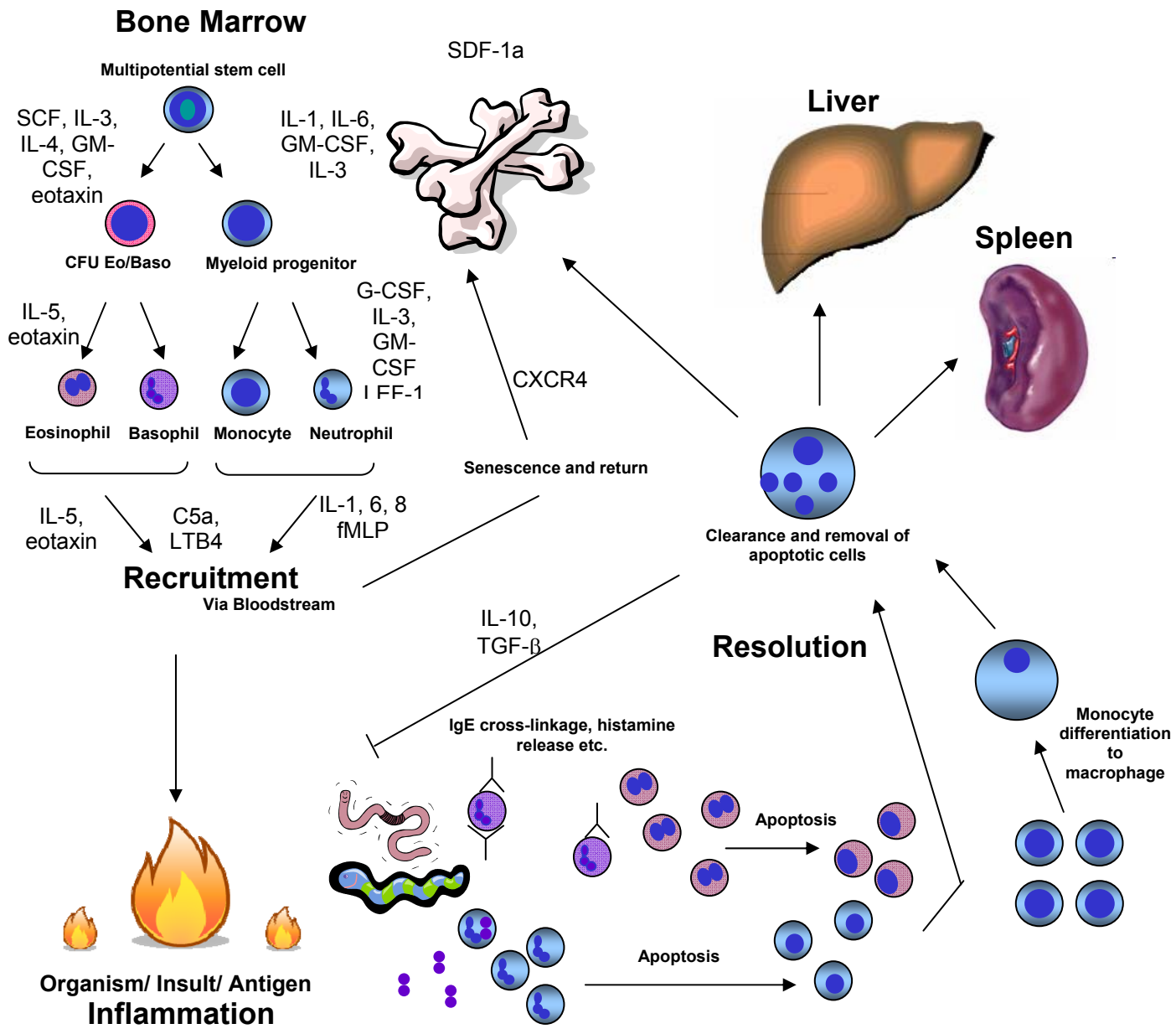


Figure 2: Granulocyte life-cycle. Under the influence of well-characterized growth factors, granulocytes are differentiated from multi-potent stem cells in the bone marrow. At the termination of normal resolving inflammation, granulocytes undergo apoptosis as a prerequisite for non-inflammatory removal by phagocytes such as macrophages. Up-regulation of the receptor CXCR4 serves as a homing beacon drawing senescent cells towards the organs responsible for their clearance, i.e. the bone marrow, liver, and spleen.

Convincing evidence from mouse knockout models has recently advanced our understanding of the key transcriptional regulatory elements involved in granulocyte differentiation and these include: NF-κB (p50 stimulates C/EBPα when inflammatory stimuli present) (Wang et al. 2009), C/EBPα, C/EBPε, Transcription factor PU.1(PU.1) and Retinoic acid receptor (RAR) for neutrophils while eosinophil differentiation is directed by NF-κB, nuclear factor of activated t-cells (NFAT) and GATA binding protein 3 (GATA-3) (Friedman 2007). As differentiation and maturation progress cells increasingly lose the ability to proliferate until in the terminally differentiated (fully mature) state they can progress only unto death. In the terminally differentiated state it seems logical to assume that cell-cycle machinery is either physically lost or functionally redundant. Researchers in the field of granulocyte biology have previously been significantly limited in their experimental options by the requirement either to use primary and therefore short-lived, non renewable cells or 'representative' cell-lines that are usually a pale imitation of the unique biology of the cells they substitute for. Recently, a number of molecular biology techniques in routine use in other cell types have been attempted using neutrophils. Antisense/siRNA techniques have never become established in neutrophil work despite some early descriptions presumably because of the sensitivity of these cells to the reagents involved and the time required to achieve an adequate transfection (Leuenroth et al. 2000b;Sivertson et al. 2007). Sabroe *et al* have now shown that they can influence neutrophil longevity by targeting the TLR_4/NF-κB pathway with lentiviral technology (Dick et al. 2009). HIV-tat transduction has also been used with some success in granulocytes (Rossi et al. 2004) and our group has previously used this technique to target the NF-κB pathway in eosinophils (Fujihara et al. 2002). Additionally, Zemans *et al*., used retroviral transduction to introduce bcl-2 into murine bone marrow-derived cells which were subsequently differentiated into neutrophils and demonstrated extended longevity (Zemans et al. 2009). Viral genetic manipulation of neutrophil precursors (indeed of any immune cell) has significant limitations so the directed development of neutrophils from pluripotent stem cells has, therefore, been an exciting and important advance. Previously, expansion and differentiation of $CD34^+$ cells towards a myeloid lineage had been attempted but these

cells were unsatisfactory in that they had limited bactericidal capacity (Dick et al. 2008). In 2009, Yokoyama *et al* published a description of functionally characterised neutrophils derived from human embryonic stem cells (Yokoyama et al. 2009). They used bone morphogenic protein-4 (BMP-4), stem cell factor (SCF), Flt-3 ligand (FL), IL-6/IL-6 receptor fusion protein (FP6), and thrombopoietin (TPO) to form embryoid bodies which were co-cultured with OP9 cells and a combination of SCF, FL, FP6, IL-3, TPO, and G-CSF. This 2 week process produced neutrophils capable of superoxide anion production, phagocytosis, bactericidal activity, and chemotaxis but with different surface antigen expression patterns (differences in CD16, CD64 and CD14 expression). This process will no doubt undergo refinement and improvement in future to allow a production-line of faithfully replicated and conserved neutrophils for *in vitro* experimentation.

The discovery that neutrophils could be driven to undergo apoptosis by pharmacological inhibitors of elements of the cell-cycle machinery termed CDKs has established a major research track for our group (Duffin et al. 2009;Leitch et al. 2009;Leitch et al. 2010a;Rossi et al. 2006). This finding was unexpected given the cell-cycle status of neutrophils as the only other terminally differentiated cell type (neurons) studied with these pharmacological agents, prior to our work, demonstrated enhanced longevity (Dhavan et al. 2001). Additionally it had been shown that some CDKs were progressively down-regulated as myeloid lineage cells matured (Klausen et al. 2004). Myeloid lineage cells differentiate in the following order: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, segmented cell, neutrophil. Klausen *et al*., using primary human bone-marrow cells from healthy volunteers described progressive loss of CDK2, 4 and 6 which began at the myelocyte/metamyelocyte stage and was almost complete by full maturation (Klausen et al. 2004). This occurrence coincided with upregulation of the endogenous CDK inhibitor p27kip1 and both results were shown at the mRNA and protein level. No consistent finding with regard to the up or down-regulation of the CDK binding partners, termed cyclins, was obtainable illustrating the complexity of regulation involved in this system. This finding was important as other works on granulopoiesis had utilised a variety of cell-lines and discovered, perhaps

unsurprisingly, a variety of CDK/CDK binding partner expression patterns. It also suggested that p27kip1 might be more important than the commonly implicated endogenous CDK inhibitor, p21. Interestingly, p21 has come to the fore recently as a novel modulator of the response to LPS made by inflammatory macrophages (Lloberas et al. 2009;Scatizzi et al. 2006;Scatizzi et al. 2009).

1.2.3 Mature granulocytes

The largest granulocyte reserve is in the bone marrow though there are pools of neutrophils at the vascular margins and in the pulmonary capillary 'sink'. Mobilisation of the bone marrow reserve occurs in response to infection/ allergen and results in significant increases in the circulating population (orders of magnitude in some cases). Granulocytes exit the bone marrow trans-cellularly across the sinusoidal endothelium. Neutrophils are retained in the bone marrow by high concentrations of SDF-1a which signals through the chemokine (C-X-C motif) receptor (CXCR)-4 (Figure 2) (Eash et al. 2009). They are stimulated to begin egress by the presence of chemotactic factors such as leukotriene (LT)-B4, C5a and IL-8 (as well as CXC chemokines KC (chemokine (C-X-C motif) ligand (CXCL)-1) and macrophage inflammatory protein (MIP-2, CXCL2) in murine models) (Wengner et al. 2008). Additionally, G-CSF has been shown to enhance mobilization as well as bone marrow production of neutrophils at resting state and may solely function by mobilization under inflammatory conditions (Panopoulos et al. 2008). The same effect in eosinophils is mediated by eotaxin and IL-5. Granulocyte exit from the bone marrow occurs via integrin-dependent processes. Once in the circulation both of the major granulocytes have half-lives from 6-12h though this may be greatly extended, in the case of eosinophils, by the presence of a pathological eosinophilia. In tissue eosinophils can survive much longer than neutrophils (up to 2 weeks) (Cara et al. 2000;Giembycz et al. 1999a).

Neutrophils are drawn to sites of inflammation by a variety of signals including formyl-methionyl-leucyl-phenylalanine (fMLP), LTB_4, IL-8, C5a, CXCL1, and CXCL5.

Eosinophils also follow chemotactic gradients of diverse stimuli including: platelet activating factor (PAF), LTB_4, C5a, eotaxin and the cytokines IL-5, TNF-α and IL-2 (Baggiolini 1998;Baggiolini et al. 2000;Petri et al. 2008;Sallusto et al. 2008). These chemotactic mediators are sensed by specialised receptors on the granulocyte plasma membrane (the majority of which are g-protein coupled receptors (GPCRs)). The detection of chemotactic stimuli allows the neutrophil to follow a gradient towards the epi-centre of inflammation. At sites of maximal stimulation the signal changes to bias neutrophils towards adhesion, transmigration and initiation of inflammatory function. A growing cohort of intracellular signalling effectors engaged by g-protein activation includes Ras- and Rho-family GTPases, PI3K, phospholipase (PL)- C and PLA2 but currently much attention has been focused on the PI3K family. Under certain conditions it has been shown that the chemotactic process can occur by polarisation of PI3K signalling, in alignment with the chemoattractant gradient, leading to cytoskeletal rearrangement (Ferguson et al. 2007;Stephens et al. 2008). Such is the diversity of signals that the neutrophil must respond to in order to maintain a comprehensive defensive response that it is perhaps not surprising that a variety of intracellular signalling responses have been identified. It appears likely that whichever intracellular signal is activated cell movement occurs in the direction of the leading edge of membrane with the strongest activation of signalling.

Granulocytes traverse the vascular endothelium into tissue by the well-described processes of rolling (CD62L dependent), adhesion (margination) and translocation (diapedesis) across the post-capillary venule wall or in the lung at capillary level (CD11b/CD18-(integrin) dependent). The process of transmigration is not well understood but is believed to occur through two routes. The first, more generally believed, is the paracellular route which occurs through small gaps at intracellular endothelial cell junctions. The second route is the transmigration pathway, where increasing morphological studies have demonstrated granulocyte migration through the endothelial cells themselves (Engelhardt et al. 2004;Fernandez-Borja et al. 2010). Which ever the mode of migration, it would seem that granulocyte crossing of the endothelium

is not a one-way system. Recent *in vitro* work by Buckley *et al*, has described a population of long-lived neutrophils which can migrate in a retrograde direction through an endothelial monolayer i.e. reverse transmigration neutrophils (RT neutrophils) which have a distinct phenotype ($CD54^{high}$, $CXCR1^{low}$) and function and are thought to be present in the peripheral circulation in humans (Buckley et al. 2006). The response involves millions of granulocytes and it is important that cells are effectively cleared so that there is no potential for undirected, self-destructive activity. This is delivered by apoptosis and macrophage driven phagocytic clearance (Savill et al. 1989), by epithelial cell phagocytosis of apoptotic cells or by mucociliary escalator clearance of cells and debris from the airway lumen. Where clearance is insufficient (i.e. under normal physiological conditions where there is still a vast daily turnover of approx 10^{11} neutrophils/24h) it has been shown that upregulation of the receptor CXCR4 serves as a homing beacon drawing senescent (pre-apoptotic) cells towards the organs responsible for their clearance (Martin et al. 2003). The liver, bone marrow and spleen split this task in a roughly equal three-way fashion (Furze et al. 2008a;Furze et al. 2008b).

1.2.4 Granulocyte apoptosis

Neutrophils and to a lesser extent eosinophils are short-lived cells, a feature that may help to limit their potential for causing damage by ensuring that they can't be subverted by pathogens. They are explosively, reactive cells and it is therefore surprising that their death is a model of contained self-restraint. Apoptosis is a physiological marvel that allows cells with an incendiary cargo (granules) to package it efficiently and safely (in plasma-membrane) so that the cellular environ is protected. In addition the cell nucleus condenses and chromatin is cleaved internucleosomally and re-organised into apoptotic bodies. Throughout this process the cell-membrane is retained intact though glycoprotein and phospholipid signals are displayed in order to attract macrophages and facilitate interaction and uptake (efferocytosis). Annexin-V is often used to label phosphatidylserine (PS) residues which are characteristically flipped to the outer-membrane of apoptotic cells thereby enabling flow cytometric identification of

apoptosis. Another member of this family, Annexin-1, has been shown to induce granulocyte apoptosis but is also released by both neutrophils and macrophages in order to enhance phagocytosis of apoptotic cells (Perretti et al. 2004;Scannell et al. 2007b). The whole process is amplified by many orders of magnitude during inflammation whether it be neutrophil- or eosinophil-dominant. Apoptosis is not a persistent state and apoptotic cells not cleared by phagocytes may then undergo secondary necrosis with deleterious consequences for surrounding tissues. Large-scale granulocyte recruitment must necessarily be followed by large scale granulocyte apoptosis and clearance by macrophages. It is extremely important that this is taken into account when attempting to drive granulocyte apoptosis in order to promote resolution of inflammation (Bianchi et al. 2006;Rossi et al. 2007).

Neutrophil and eosinophil apoptosis are similar but not identical processes. It is generally believed, though not universally (Maianski et al. 2003) that there are two pathways by which apoptosis proceeds both of which are ultimately dependent on the caspase family (Figure 3). The intrinsic pathway occurs when the cell is subjected to withdrawal of growth/survival factors, genotoxic stress or ultraviolet (UV)-irradiation. This pathway relies on pro-apoptotic members of the B-cell lymphoma (Bcl)-2 family which escape regulation by their anti-apoptotic counterparts and translocate to the mitochondria facilitating liberation of cytochrome-c. The various components of the apoptosome (Apaf-1, cytochrome-c etc.) then assemble to cleave the inactive zymogen, procaspase-9, to the active initiator caspase-9 which commits the cell to executioner caspase-3 mediated apoptosis. The extrinsic pathway proceeds via external cell-membrane death-receptors such as the TNF receptor (TNFR), the Fas receptor (FasR) and TNF-related apoptosis-inducing ligand receptor (TRAILR). Ligand activation of receptors promotes clustering and enhances association with the internal adaptor proteins (Tumour necrosis factor receptor type 1-associated death domain (TRADD) and fas receptor associated death domain (FADD)) within the lipid-raft. Multiple procaspase-8 molecules assemble at the adaptor proteins (formation of the death inducing signal (DISC) complex) and their physical approximation generates an

autocatalytic reaction initiating the caspase-cascade. The death-receptor CD137 has also been implicated in neutrophil and eosinophil apoptosis but it is currently unclear what its physiological role is (Simon 2003).

To further complicate the story it is apparent that there is a degree of cross-talk between the intrinsic and extrinsic pathways. Caspase-8 may alternatively cleave BH3 interacting domain death agonist (Bid) which can translocate to the mitochondrial-membrane and permeabilise it allowing apoptosis to proceed via intrinsic pathway apoptotic machinery (Bianchi et al. 2006;Simon 2003).

Eosinophil apoptosis most likely occurs along approximately the same lines, however controversy still reigns with regard to the caspase family. It has previously been stated that caspases-3, -8 and -9 have no demonstrable role in eosinophil apoptosis (Daigle et al. 2001b). Subsequently, it has been suggested that perhaps caspase-9 plays a role and that therefore the mitochondrial pathway is of importance (Peachman et al. 2001;Simon 2001). This work was confirmed by observations with regard to the effects of known eosinophil apoptosis-inducing agents such as glucocorticoids which appear to mediate their effects via mitochondrial permeabilisation with caspase activation merely a downstream effect. By contrast, Fas ligation has been shown to promote eosinophil apoptosis in a caspase-3 and -8 dependent manner and although mitochondrial integrity was disrupted it proved non-essential for apoptotic progression (Daigle et al. 2001a;Simon et al. 1999;Simon 2001;Simon 2006).

Support for the predominance of mitochondria-driven granulocyte apoptosis is provided by the increasing evidence that survival proteins are key determinants of neutrophil longevity. The anti-apoptotic Bcl-2 family member, Mcl-1, is present in both neutrophils and eosinophils but other family members show contrasting expression. Neutrophils express A1 (Bfl-1) while eosinophils favour Bcl-extra long (xl) and Bcl-2 itself appears not to be constitutively present at demonstrable levels. Interestingly however, in eosinophils Bcl-2 expression can be stimulated by IL-5 (Ochiai et al. 1997). These

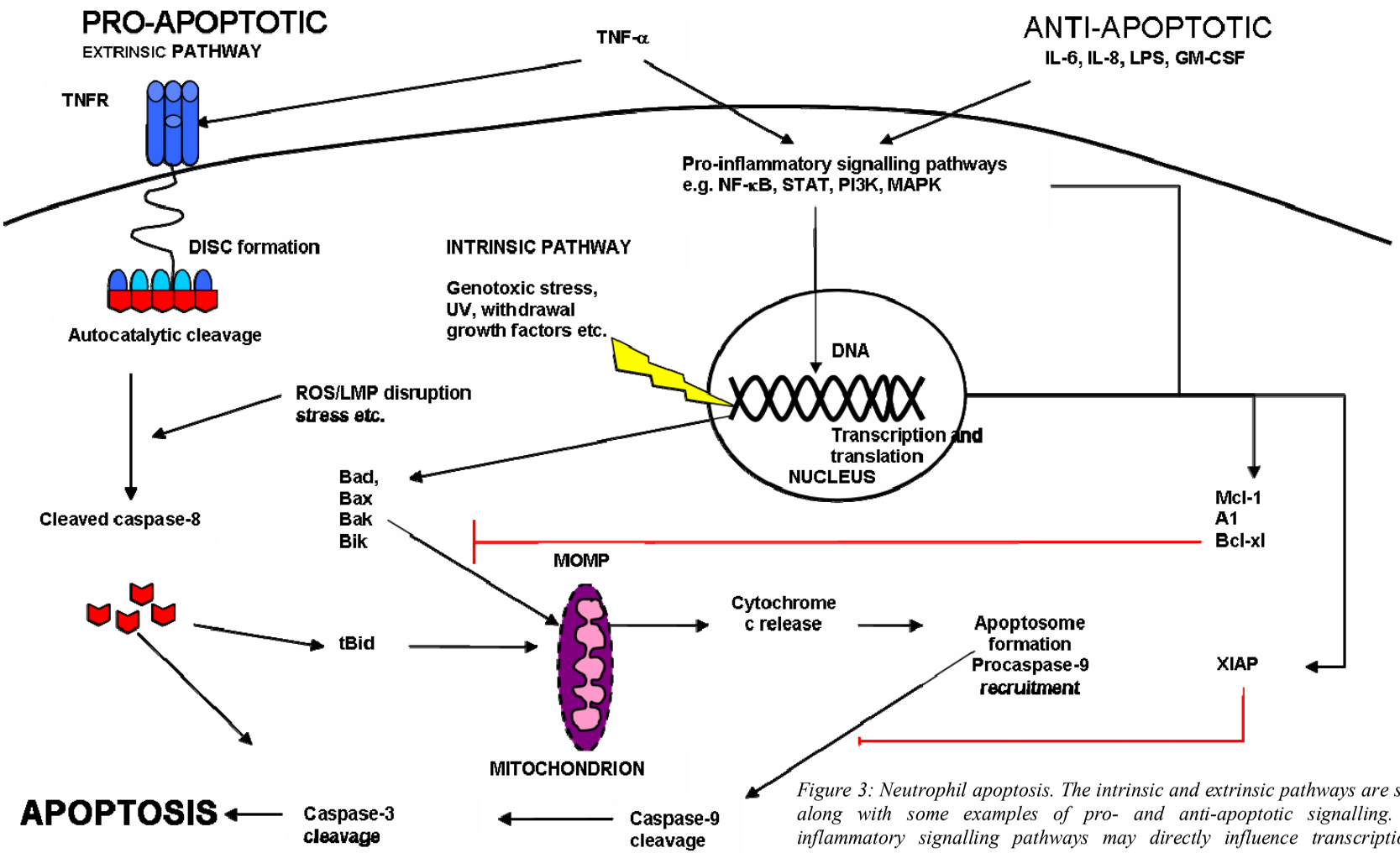


Figure 3: Neutrophil apoptosis. The intrinsic and extrinsic pathways are shown along with some examples of pro- and anti-apoptotic signalling. Pro-inflammatory signalling pathways may directly influence transcription of survival proteins e.g. NF-κB promotes transcription of XIAP or increase protein stability e.g. ERK (of the MAPK family) maintains XIAP levels. TNF-α may have both early pro-apoptotic action and late anti-apoptotic effects. TNFR is the TNF receptor. DISC refers to the Death Inducing Signalling Complex which is composed of TNFR-associated death domain protein (TRADD), Fas-associated death domain protein (FADD) and procaspase-8. MOMP is mitochondrial outer membrane permeabilisation

survival proteins mediate their effects by marshalling pro-apoptotic Bcl-2 family counterparts away from the mitochondrial-membrane. Pro-apoptotic Bcl-2 family members are capable of causing permeabilisation of the outer mitochondrial-membrane (MOMP) when numbers predominate over those of their chaperones. Bcl-2-(associated X) protein (Bax) is perhaps the best characterised member of this family and appears to play a pivotal role in neutrophil apoptosis. In eosinophils however Bax fails to be down-regulated by anti-apoptotic survival factors weakening its position as a key player in apoptosis (Weinmann et al. 1999). The role of Mcl-1 in neutrophil apoptosis has received a great deal of attention and it appears that this protein is an essential component of neutrophil viability. A contention that is supported by the neutropenic phenotype of the *Mcl-1* knockout mouse in comparison with the increased apoptotic phenotype of the *A1* knockout mouse (Dzhagalov et al. 2007b;Hamasaki et al. 1998). Additionally the pro-apoptotic protein BCL2-like 11 (apoptosis facilitator) (Bim) has recently been described as having a prominent role in neutrophil death following cytokine stimulation and is additionally implicated as a known binding partner of Mcl-1 (Andina et al. 2009;Cowburn et al. 2010;Herrant et al. 2004;Villunger et al. 2003).

1.2.5 Modulation of granulocyte apoptosis

Granulocyte longevity is necessarily highly regulated and consequently highly variable from a matter of hours up to 2 days for neutrophils and extending to 2 weeks for eosinophils. Persistent markers of bacterial infection such as LPS, pathogen derived molecules that act as ligands for TLR 2, 4 and 9 and inflammatory cytokines such as IL-6, IL-8, GM-CSF and TNF-α can delay neutrophil apoptosis whilst resolving infection indicated by successful neutrophil phagocytosis of bacteria, removal of bacterial products and down-regulation of inflammatory cytokines will promote apoptosis (Sabroe et al. 2005;Sabroe et al. 2007b).

Granulocyte longevity is also extremely dependent on intracellular signalling pathways. Perhaps the most important of which is that controlled by the pivotal transcription factor

of inflammatory cytokines NF-κB. NF-κB can be activated by LPS and is known to control the survival proteins X-linked inhibitor of apoptosis protein (XIAP) (an inhibitor of caspase-3, -8 and -9) and Bcl-xl thus enhancing neutrophil and eosinophil longevity by tangible means. Pharmacological inhibitors of NF-κB such as gliotoxin promote neutrophil apoptosis and in combination with TNF-α cause dramatically enhanced apoptosis. This feature may partially explain why TNF-α has opposing effects on neutrophils at early and late time-points (Lawrence et al. 2001;Ward et al. 1999). Other important signalling pathways include the phosphoinositide 3-kinase (PI3K) pathway which can be stimulated by GM-CSF to promote longevity by upregulation of Mcl-1 in neutrophils and which has an emerging role in the maintenance of eosinophilic inflammation (Pinho et al. 2005;Pinho et al. 2007). Similarly the extracellular signal regulated kinase (ERK) pathway is likely to be important for survival factor mediated anti-apoptotic effects at sites of inflammation. However, direct inhibition of either of these pathways will not promote granulocyte apoptosis per se (Rossi et al. 2007;Savill et al. 1995).

Perhaps the most interesting and clinically relevant modulation of granulocyte apoptosis is that achieved with the use of glucocorticosteroids. These drugs extend neutrophil life-span but promote eosinophil apoptosis. This effect seems to correlate with their ability to up-regulate Mcl-1 in neutrophils but down-regulate the same protein in eosinophils (Sivertson et al. 2007). Dexamethasone (a stereotypical glucocorticosteroid) has also been shown to promote macrophage phagocytosis of apoptotic neutrophils (Heasman et al. 2003). The impact of putative inflammation resolving agents on clearance of apoptotic neutrophils is of paramount importance as prompt removal is essential to avoid secondary necrosis and loss of toxic contents. This is perhaps why the discovery of a new class of endogenous lipid derived pro-resolution agents has been so exciting. The lipoxin family produced by neutrophils and macrophages limit the recruitment of neutrophils to inflammatory sites and enhance macrophage phagocytosis of apoptotic neutrophils. This occurs as part of a natural brake on progression of inflammation and there is hope that enhancement of this pathway may provide a novel therapeutic strategy

to counter inflammatory disease, a hypothesis that has already shown promise in several animal models (Serhan 2007). Another exciting prospect for therapeutic modulation of granulocyte apoptosis has emerged with the discovery of active cell-cycle machinery in neutrophils which can be inhibited to promote apoptosis. CDK inhibitor drugs promote neutrophil apoptosis and drive resolution of inflammation in animal models. Given that these drugs are already in use for the treatment of cancer it is possible that they could make the transition from bench to bedside for the management of inflammatory disease in the near future (Hallett et al. 2008a;Rossi et al. 2006).

1.2.6 Alveolar macrophages and phagocytic clearance of apoptotic granulocytes

In normal healthy lungs the predominant alveolar leucocyte is the macrophage, a cell that becomes resident following differentiation from a bone-marrow produced circulating pre-cursor monocyte. Alveolar macrophages occupy a unique position at the interface between inhaled air (and hence the external environment) and the circulation (in the form of the alveolar capillary network which is closely juxtaposed to the alveolus to promote efficient gaseous exchange) and because key macrophage functions include chemotaxis, phagocytosis and cytotoxicity they are lynch-pins of immune defence. Importantly, during pulmonary infective or inflammatory disease a key role in resolution of inflammation falls to both alveolar and recruited circulating macrophages. These cells are responsible for the removal of apoptotic neutrophils and to some extent eosinophils (though epithelial cells may be equally important for eosinophil removal) by efferocytosis (see figure 4 for an example of experimental quantification of this process). In order for an apoptotic cell to be recognized by a macrophage it must display specific signals. The most obvious change in the apoptotic granulocyte's plasma membrane is the externalization of PS residues but this in itself is not sufficient to expedite phagocytosis. The search for a macrophage docking receptor on granulocytes has been exhaustive but inconclusive and currently a combination of various glycoproteins and phospholipids are of postulated importance. These putative receptors include the recently identified Tim4

(Kobayashi et al. 2007), stabilin-2 (Park et al. 2008), and BAI1 (Park et al. 2007). The process of efferocytosis, literally meaning 'burying the dead', is a pro-resolution strategy in itself. Macrophages that consume apoptotic neutrophils switch to a resolution phenotype that allows them to secrete transforming growth factor (TGF)-β and IL-10 (Fadok et al. 1998) as opposed to pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α. This is in contrast to macrophages that have consumed necrotic neutrophils where the opposite is true and inflammation is actively propagated. Additionally, the pro-resolution phenotype includes the production and enhanced responsiveness to lipoxins, protectins and resolvins. These mediators enhance macrophage phagocytosis and promote pro-resolution cytokine production (Serhan 2007).

Figure 4: Macrophage phagocytosis of apoptotic neutrophils

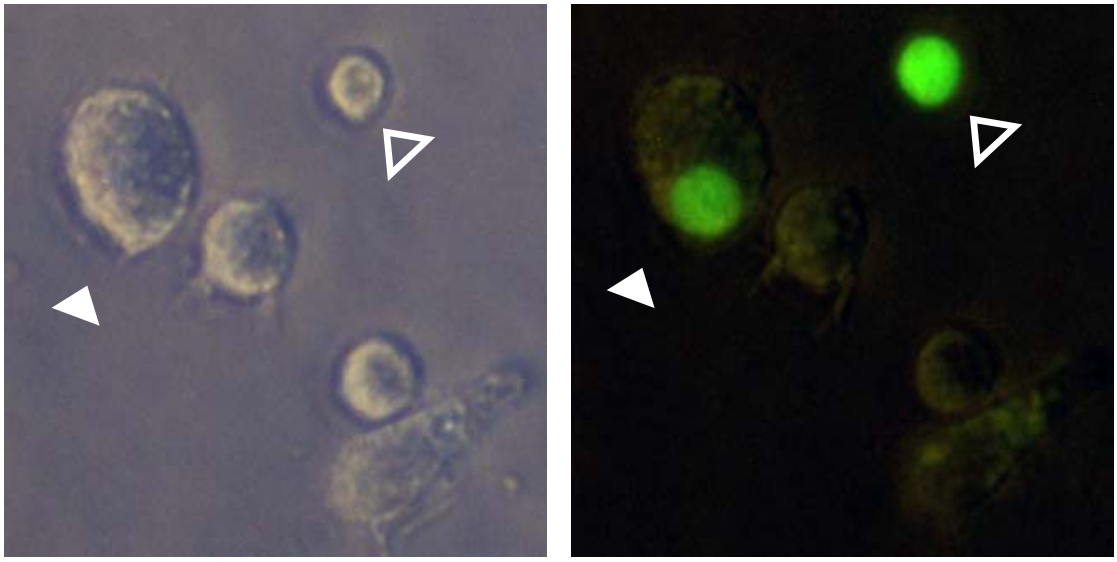


Figure 4: In vitro phagocytosis experiment with human monocyte derived macrophages and CellTracker™ green labelled apoptotic neutrophils. *Fluorescence microscopy- phase image on left and UV on right. Solid arrow shows macrophage that has ingested a neutrophil. Empty arrow shows apoptotic neutrophil. Bottom right macrophage may have already digested a labelled neutrophil*

1.2.7 Granulocytes and disease

There has been a tendency, certainly in clinical circles to question the validity of inflammation-based hypotheses for various diseases based on the efficacy or lack of efficacy of glucocorticosteroid medications. Given the complexity of the inflammatory response and the relative bluntness of this therapeutic tool it is overly simplistic to make such assumptions. Steroid medications have certainly been a paradigmatic therapy in the treatment of inflammatory disease but they do not and will not drive resolution of inflammation in all settings. With increased understanding of the mechanisms of the inflammatory response and a new focus on its resolution it is hoped that novel incisive or pleiotropic therapy combinations may be developed to address the inflammatory lung diseases discussed below.

COPD

In most cases of COPD (α-1-antitrypsin deficient patients are a notable exception) the respiratory mucosa is damaged by repeated exposure to inhaled toxic chemicals leading to chronic inflammation, reduced immunity and susceptibility to respiratory infections. COPD is a prevalent, largely smoking-related (though there is an increasingly recognised occupational contribution) disease in this country (in others it is related to the burning of bio-fuels) which presents with increasing breathlessness and a productive cough. It is an obstructive airways disease but unlike asthma this obstruction is usually irreversible with inhaled therapies. The mainstay of current treatment is with inhaled or oral corticosteroids and antibiotics when exacerbations are judged infective (BTS 1997;MacNee et al. 2003).

Neutrophils are likely to play an important role in this condition and they are found in increased numbers throughout the respiratory tract. The highest concentrations of neutrophils are found in sputum and bronchoalveolar lavage fluid (BAL) (which is perhaps representative of rapid airway directed migration) but numbers of neutrophils are also increased in lung parenchyma and airway smooth muscle. Neutrophilic inflammation is characteristic of COPD exacerbations and there is a correlation between

the resting burden of lung based neutrophils and severity of disease phenotype. Neutrophilic inflammation appears to be driven by the irritant force as smoking drives alveolar macrophages and epithelial cells to express increased levels of IL-8, a potent neutrophil chemoattractant. IL-8 also stimulates neutrophils to release MPO while TNF-α and LTB_4 (produced by epithelial cells, mast cells and T-lymphocytes) cause neutrophil activation (degranulation, ROS production and exocytosis of lysosomal enzymes) (MacNee 2007;O'Donnell et al. 2006a;O'Donnell et al. 2006b). Meanwhile macrophage phagocytic function is impaired by cigarette smoking and cannot keep pace with the increased neutrophil burden (Kirkham et al. 2004). Enhanced neutrophil recruitment and activity combined with decreased macrophage phagocytosis weigh the scales heavily towards neutrophil mediated tissue damage. COPD lungs are subjected to high levels of toxic neutrophil products including: ROS, elastase and proteinases. These toxic substances overload the capacity of native antiproteinases and antioxidants to neutralize them which leads to damaged epithelial cilia and decreased mucociliary clearance. Damage to the respiratory mucosa means that alveolar cells are replaced by goblet cells which increase mucous production (see figure 1). Furthermore, airways are progressively remodelled by the reparative process so that they become thicker, less efficient conductors of air. There is some controversy with regard to the longevity of neutrophils isolated from the airways of COPD patients. Studies have shown both enhanced longevity and no enhancement of longevity while peripheral blood neutrophils appear to have an increased lifespan in keeping with systemic inflammation (Barnes 2007;Churg et al. 2008;Quint et al. 2007a).

COPD is notoriously resistant to glucocorticoid therapy perhaps because these drugs promote neutrophil longevity by upregulating the survival protein Mcl-1 (Sivertson et al. 2007). It is also known that smoking promotes dysfunction of histone deacetylase (HDAC) 2 an enzyme which is usually recruited by glucocorticoid receptors to switch off the transcription of pro-inflammatory genes (Ito et al. 2006;Kagoshima et al. 2003). The small benefits attributable to steroid therapy in COPD may stem from enhancement of macrophage phagocytosis of apoptotic cells. Of other therapies currently in use

theophylline (a non-specific adenosine antagonist and phosphodiesterase inhibitor) is known to restore HDAC2 function, reversing corticosteroid resistance and reducing IL-8 concentrations and sputum neutrophilia (Barnes et al. 2004). Long acting β_2 agonists (originally employed to relax smooth muscle in peripheral airways) have now been shown to inhibit neutrophilic inflammation as measured by sputum or BAL analysis and they are reported to drive neutrophil apoptosis though the circumstances of this are difficult to ascertain. It is clear that potential therapies must consider targeting neutrophil recruitment as well as striving to drive neutrophil apoptosis and removal (Barnes 2004;Barnes et al. 2004;Barnes 2007;Quint et al. 2007b).

Idiopathic Pulmonary Fibrosis

IPF is a condition characterized by the devastation of the respiratory mucosa though its pathophysiology is complex. There is a great deal of debate about the relative importance of inflammation versus aberrant wound repair in the pathogenesis of IPF but there is sufficient evidence to justify an argument in support of a significant neutrophil-derived component to the disease model (Collard et al. 2007b;du Bois et al. 2007b;Hunninghake et al. 2007;Seaton 2000;Strieter 2008;Wallace et al. 2007)).

The argument is that the initial and subsequent 'exacerbatory' insults are acute and inflammatory even if the characteristic phenotype is conveyed by a disordered 'healing' process. Healing by fibrosis results in significant loss of lung architecture and vital respiratory mucosa to scarring (Wallace et al. 2007). Neutrophils are known to be present in BAL from IPF patients and are also significantly increased within lung tissue (Kinder et al. 2008). Additionally there is evidence of the toxic chemicals produced by neutrophils including MPO, elastase, collagenases and proteases (Haslett 1999;Hunninghake et al. 2007). The importance of this chemical insult is supported by the elastase knockout mouse which is significantly protected against lung injury and also fails to up-regulate TGF-β (Chua et al. 2007). A recent clinical paper has shown that significant neutrophilia within the BAL fluid of IPF patients correlates with increased mortality (Kinder et al. 2008). It has been argued that the terminal scarring process of

IPF occurs because of a significant loss of lung architecture and that where lung architecture is preserved there is potential for reversal of the remodeling process and resolution (Wallace et al. 2007). It has also been suggested that the fibrotic response may in a sense be driven by a persistent but ineffective pro-resolution of inflammation phenotype. This hypothesis is drawn from the observation that TGF-β is a key pro-resolution molecule but is also intimately involved in fibrogenesis. In a disease model where non-resolving low level inflammation results in sustained TGF-β production it is possible to conceive that a fibrotic healing phenotype might evolve. Therapeutic strategies that drive neutrophils towards apoptosis will certainly have something to add to treatment of this disease if they can prevent or ameliorate the inflammatory insult (whether it be acute, chronic or relapsing) that must be responsible for such extreme distortion of lung architecture (Collard et al. 2007a;du et al. 2007a).

ARDS/ALI

ARDS/ALI is the pulmonary component of the multi-organ dysfunction syndrome (MODS) and represents a global failing of lung function in response to a specific, non-specific or unidentifiable stimulus. Effectively these conditions are the result of an aggressive mucosal inflammatory immune response (Abraham 2003;Bellingan 2002;Leaver et al. 2007;Martin 2008;Matthay et al. 2005;Seaton 2000).

Characterised by the pathological reaction termed diffuse alveolar damage (DAD), ARDS/ALI seems to be a neutrophil-dominant disease (interestingly DAD is also seen in the terminal stages of IPF (Tiitto et al. 2006)). Analysis of BAL fluid from patients with early-stage ARDS demonstrates increased numbers of activated neutrophils and their numbers correlate with severity of lung injury. Indeed persistence of BAL neutrophilia at day 7 is associated with increased mortality (Steinberg et al. 1994). The pulmonary circulation contains a large sequestered neutrophil population termed the ‘marginated’ pool that does not normally circulate but is loosely adhered to the vessel walls (Summers et al. 2010). This population can be mobilized into the circulation by for example steroid therapy or exercise. Neutrophils are subject to slow transit through the pulmonary microvasculature where blood vessel diameters are smaller than their own.

The ability of neutrophils to progress depends on their considerable properties of distensibility. In ARDS/ALI neutrophils are recruited early in large numbers and an alteration in their rheological properties (they become less deformable) means they struggle to manoeuvre through vessels while cytokine excess encourages their adhesion to vessel walls and subsequent translocation into the lung parenchyma and airways. The influx of huge numbers of neutrophils and proteinaceous inflammatory oedema fluid from permeabilised vessels impairs gas exchange and makes adequate ventilation extremely difficult (Martin 2008;Matthay et al. 2005;Summers et al. 2010).

Neutrophil influx is driven by alveolar macrophage production of IL-8 and excessive levels of this cytokine have been found to be predictive of progression to ARDS in susceptible patients (Donnelly et al. 1993). There is no effective, specific therapy for this condition and yet some patients survive with supportive therapy alone, implying that successful resolution is possible and can be mediated by physiological mechanisms. Neutrophils isolated from the BAL of ARDS patients have enhanced longevity, are activated and may cause insurmountable damage to the respiratory mucosa leading to healing by fibrosis and permanent scarring with loss of lung architecture and function. In experimental models where neutrophils are depleted ALI caused by endotoxin is attenuated. In other studies enhancement of neutrophil apoptosis also reduced inflammatory parameters and tissue damage. The picture is complicated by apparent ARDS/ALI in neutropenic human patients. It is also clear that given the extravagant neutrophil influx associated with this condition it would likely require a dual strategy of enhancing both neutrophil apoptosis and neutrophil clearance to ensure that secondary necrosis (and tissue damage) is prevented by prompt removal (Abraham 2003;Oeckler et al. 2007) .

Pneumonia

Pneumonia is the term given to an infection within the lower respiratory tract that is sufficiently significant to result in visible changes on a chest radiograph (BTS 2001). There are many potential causative organisms including viruses and bacteria. Invading

pathogens that overcome immediate host defences are nonetheless recognized as foreign by their non-host constituents such as LPS or formylated peptides in their outer membranes. TLRs, CD14 and GPCRs (e.g. FMCPR) found on macrophages and other respiratory mucosal cells are responsible for this detection and subsequently stimulate an acute inflammatory reaction by the production of cytokines such as TNF-α, IL-1, IL-6 and IL-8. This pro-inflammatory milieu prolongs neutrophil lifespan and promotes neutrophil activation in order to allow effective microbial phagocytosis and killing (Droemann et al. 2000;Sabroe et al. 2005;Sabroe et al. 2007b). If the inflammatory reaction is successful in containing the pathogens and they are cleared by phagocytosis or killed then the inflammatory reaction remains localized to a single lobe or segment of the lung. The upregulation of anti-inflammatory, pro-resolution molecules should ensure containment of inflammation. Neutrophil phagocytosis of bacteria and the induction of pro-resolution cytokines such as IL-10 and transforming growth factor (TGF)-β promote neutrophil apoptosis and removal by macrophages (Watson et al. 1996). The alternative is non-resolution with enhanced neutrophil longevity and local tissue damage (resulting in abscess, empyema or bronchiectasis) and/or loss of containment resulting in global lung inflammation characterized by ARDS or spread via the circulation to other organs (sepsis) with the potential for multi-organ dysfunction syndrome (MODS). Streptococcal pneumonia (the pathogen responsible is *S.pneumoniae*) is often cited as a paradigm of resolving inflammation as despite an acute inflammatory response characterized by a massive neutrophil influx (giving the pathological appearance referred to as 'red hepatisation') it is possible for the lung to achieve complete recovery (Haslett 1999). There is however a sub-population of severe pneumonia that behaves aggressively or is not contained by the patient's immune system and antibiotic therapy resulting in admission to intensive care and potentially MODS, sepsis, terminal decline and death.

Antibiotics are central to the treatment of pneumonia but they are not always sufficient and it is possible that addressing acute inflammation associated with pneumonia may prove a successful adjuvant therapy. In this setting it is unclear whether the ideal strategy is to dampen down the inflammatory response or to augment it in the hope that

a supra-physiological immune reaction will prove successful in eradicating the organism responsible. Glucocorticoid therapy has been used as an adjuvant to antibiotics in this kind of patient with varying success rates in different trials (Cazzola et al. 2005). The theory is that steroids should dampen inflammation through down-regulation of NF-κB activation and consequently the inflammatory cytokines under its direct transcriptional control. A recent study that utilized a physiological steroid dose (Supra-physiological doses had been employed in previous studies) has shown a significant reduction in length of hospital stay and mortality in ICU patients with severe community acquired pneumonia (Confalonieri et al. 2005). It is possible therefore that in a sub-group of patients with pneumonia strategies to aid resolution of inflammation (in combination with antibiotic therapy) may be warranted.

Cystic Fibrosis

CF is the commonest inherited disease in Caucasian populations affecting 1 in 2500 births. The defect in salt transport (the genetic abnormality affects the cystic fibrosis transmembrane regulator gene (CFTR) which encodes a chloride channel) should be responsible for such widespread organ pathology including pancreatic insufficiency, bronchiectasis, liver dysfunction, infertility and gut defects (Doring et al. 2009). Mortality (on average at age 34 in Western society) is most frequently from respiratory failure caused by aggressive bronchiectasis (Seaton 2000).

There are competing hypotheses as to the root cause of the repeated infective exacerbations that lead to bronchiectasis in CF. It is postulated that a relative dehydration of the respiratory mucosa means that the mucociliary escalator is compromised by viscid mucus and that altered mucosal pH results in malfunction of host antimicrobial peptides. There may also be a failure of normal bacterial internalisation processes and it has been suggested that an intrinsic pro-inflammatory phenotype is conferred by CFTR malfunction. The pro-inflammatory phenotype theory postulates that an over-burdened endosomal system encumbered by the processing of faulty CFTR signals distress which leads to activation of NF-κB (Pahl et al. 1995;Venkatakrishnan et

al. 2000). Production of IL-8 is stimulated resulting in enhanced neutrophil recruitment. Regardless, the net result is a failure of mucosal immunity and a succession of bacterial infections occur (initially, *Staphylococcus aureus* and/or *Haemophilus influenzae* but subsequently and devastatingly *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia*). With bacterial colonization innate defence is roused and a significant neutrophil influx occurs. This is usually thwarted, initially by microbial resistance and subsequently by distorted lung architecture. This results in an insurmountable accumulation of inflammatory cells and toxic damage from chemicals eluted by their necrotic carcasses. Elastase, ROS and myeloperoxidase cause further lung damage and the clearance of inflammatory cells is so poor that the lungs literally become clogged with inflammatory cell DNA. Eventually there is a terminal paucity of functional gas-exchange equipment and respiratory failure and death supervene if lung transplant is not possible (Elizur et al. 2008b) (Banner et al. 2009;Downey et al. 2009).

Recent work on *P. aeruginosa* has provided an interesting insight into the mechanisms by which this organism thwarts immune defence. It was known that *P. aeruginosa* could effectively hide from the immune system in a protective and impenetrable bio-film. A more subtle effect mediated by pseudomonas produced pyocyanin has now been recognized. This chemical promotes neutrophil death but inhibits macrophage clearance resulting in neutrophil death by necrosis. This leads to the spillage of cathepsin G and elastase which can cleave the important chemokine receptor CXCR1 on other neutrophils. This receptor would usually allow IL-8 mediated enhancement of neutrophil killing prowess which enables neutrophils to kill pseudomonas. In CF patients neutrophils have little CXCR1 for the above reason and are consequently effectively crippled as well as out-manoeuvred. Even more detrimental, fragments of this receptor stimulate the TLR system resulting in enhanced neutrophil recruitment and prolongation of the inflammatory response (Sabroe et al. 2007a).

Anti-inflammatory therapy in the form of non-steroidal anti-inflammatory drugs (NSAIDs) has been used to mild benefit in CF but it is appears that the early

introduction of a potent anti-inflammatory pro-resolution therapeutic strategy might be of enhanced benefit. Neutrophils in CF patients are dysfunctional and ineffective at restraining or removing typical CF pathogens so driving their apoptosis and removal might be of more benefit than allowing them to remain in the hope that they are contributing to defence against these microbes (Elizur et al. 2008a).

1.3 Mcl-1, a key neutrophil survival protein

In order to drive neutrophil apoptosis it is important to understand the mechanisms responsible for neutrophil survival. Potentially the most important neutrophil survival protein is Mcl-1 (Edwards et al. 2004a) as evidenced by studies utilising anti-sense Mcl-1 RNA to knockout Mcl-1 in neutrophils and most recently by the Mcl-1 myeloid-specific knockout mouse which was effectively neutropenic (Dzhagalov et al. 2007a;Leuenroth et al. 2000b).

1.3.1 Mcl-1 structure and function

The Mcl-1 gene is located on chromosome 1q21 and encodes a 40kDa protein. Mcl-1 is a member of the bcl-2 homologue family. Mcl-1 has BH domains 1-3 but in common with the A1 protein is missing the putative protein interaction domain BH4. Bcl-2 the prototype for the family is a much smaller protein at 26kDa. This is because Mcl-1 contains multiple domains rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) in its C-terminal tail that target it for degradation by the proteasome. It also possesses 2 key asparagine sites that may function as substrate for caspase cleavage (Akgul et al. 2000;Mandelin et al. 2007). Thus Mcl-1 has an extremely short half-life between 0.5 and 3h though obviously this can vary considerably dependent on cell-type and conditions. Mcl-1 was originally identified in the myeloid leukaemia myeloblastic cell line (ML-1) and was discovered to confer survival on various cell types. It has been noted that though Mcl-1 is expressed across a wide range of tissues it is most likely to be of functional importance where Bcl-2 is not expressed or

only minimally expressed (Akgul et al. 2000;Mandelin et al. 2007). Indeed in cells with little bcl-2 (e.g., the neutrophil) Mcl-1 tends to be expressed to a greater extent. Mcl-1 is known to be of particular importance in the early differentiation of myeloid cells. As is the case for many short-lived survival proteins Mcl-1 has been found to be significantly upregulated in various cancers (Ding et al. 2007;MacCallum et al. 2005;Moulding et al. 2000;Raje et al. 2005;Yoon et al. 2002). The pro-survival effect conferred by Mcl-1 is mediated by interactions with the pro-apoptotic Bcl-2 homologues Bim, Bid, Bax and Bcl2-antagonist/killer-1 (Bak). Mcl-1 has been shown to directly interact with Bid, Bak and Bim and to sequester them in an inactive form which prevents association with the outer mitochondrial membrane, pore-formation and progression down the intrinsic apoptotic death pathway (Chen et al. 2005;Han et al. 2004;Hutcheson et al. 2005;Zhu et al. 2004). Bim is likely responsible for the activation of Bax and to some extent Bak suggesting that Mcl-1 perhaps has indirect effects on these proteins. It has certainly been shown that double knockouts of Bim and Bax/Bak have enhanced longevity compared to single knockouts of any one of these pro-apoptotic bcl-2 homologues (Hutcheson et al. 2005). There is increasing evidence for the importance of Bim in the regulation of neutrophil apoptosis and it seems increasingly likely that neutrophil survival is most dependent on the balance between Mcl-1 and Bim (Andina et al. 2009;Cowburn et al. 2010;Villunger et al. 2003). This is likely to be especially true at sites of inflammation as Bim has been shown to be induced by cytokines (hypothetically as a brake on cytokine-induced longevity (Andina et al. 2009)) while Mcl-1 has been shown to be upregulated in neutrophils from the inflamed joints of rheumatoid arthritis sufferers.

1.3.2 Mcl-1 transcription and post-transcriptional regulation

There is evidence to suggest that Mcl-1 may be upregulated by a number of cytokines relevant to the inflammatory milieu including: TNF-α, IL-1b, IL-6 and GM-CSF (Liu et al. 2005). A requirement for integration of these varied signalling pathways suggests the involvement of transcription factors and potential binding sites for NF-κB, signal transducer and activator of transcription (STAT), steroid regulatory element (SRE),

cAMP responsive element binding protein (CREB) binding protein (BP) and v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (Ets) have been identified at the 5' flanking region of the gene transcription start site (Akgul et al. 2000). The functional significance of these sites, especially the NF-κB site is debated but regardless rapid up or down-regulation of transcription in the presence of various agents and cytokines has been documented. Mcl-1 is subject to alternative splicing and it has been suggested that the exon-1 variant may have pro-apoptotic effects (Bingle et al. 2000). Post-transcription Mcl-1 is subject to phosphorylation at various sites including ERK-mediated phosphorylation of Threonine 163 (Derouet et al. 2004) which serves to stabilise the protein and glycogen synthase kinase (GSK)-3 (itself inhibitied by v-akt murine thymoma viral oncogene homolog 1 (Akt-1) phosphorylation) phosphorylation at Serine 159 (Maurer et al. 2006) which enhances its proteasomal degradation. In addition the ubiquitin ligase, HECT, UBA and WWE domain containing-1(MULE) may be responsible for polyubiquitination of Mcl-1 which targets it to the proteasome (Zhong et al. 2005). Another pro-apoptotic variant of Mcl-1 may be created by caspase 3 or 8 mediated cleavage at conserved Asp residues with studies suggesting that the resultant fragments actively drive apoptosis as opposed to merely being a silent part of the process (Derouet et al. 2006;Herrant et al. 2004). Granzyme B (Han et al. 2004), TRAIL (Han et al. 2006), TNF-α (Cross et al. 2008), sodium salicylate (Derouet et al. 2006), okadaic acid (Derouet et al. 2006) and CDK inhibition (Leitch et al. 2010b;Rossi et al. 2006) have all been shown to downregulate Mcl-1 whilst dexamethasone (Sivertson et al. 2007), GM-CSF (Derouet et al. 2004) and proteasome inhibitors (Edwards et al. 2004b) are known to stabilise it.

1.3.3 Mcl-1 and signalling pathways

In the preceding section the regulation of Mcl-1 by GSK-3 and ERK was mentioned but other signalling pathways have also been implicated. Given that the best characterised neutrophil survival pathway is the NF-κB signalling system it would seem logical that there should be a link between it and Mcl-1. Although there is a transcription factor

binding site for NF-κB at the Mcl-1 promoter it has been contended that is not functional (Akgul et al. 2000). Indeed it has been suggested that the effects of traditional stimulants of the NF-κB pathway on Mcl-1 may in fact be mediated by PI3K mediated phosphorylation of Akt (Epling-Burnette et al. 2001;Liu et al. 2001). This not only prevents GSK-3 destabilisation of Mcl-1 but may promote Mcl-1 transcription by an interaction with the transcription factor CREB. Additionally, the Janus kinase (JAK)/STAT pathway has been shown to be important for the expression of Mcl-1 induced in neutrophils by GM-CSF (Derouet et al. 2004) while STAT-3 has a key role in Mcl-1 expression in macrophages (Liu et al. 2003). Epidermal growth factor (EGF)/ v-raf-1 murine leukemia viral oncogene homolog-1(Raf 1) and p38 mitogen associated protein kinase (MAPK) have also been shown to regulate Mcl-1 under specified conditions (Saffar et al. 2008;Yoon et al. 2002).

Mcl-1 is subject to rapid up or down-regulation of transcription in response to inflammatory cytokines, a short half-life and extensive post-trancriptional regulation. This makes Mcl-1 an ideal 'survival switch' for the neutrophil population it allows enhanced longevity at times of need but ensures prompt turnover of unrequired cells.

1.4 Cyclin-dependent kinases, CDK inhibitor drugs and resolution of inflammation

1.4.1 Cyclin-dependent kinases: structure and function

CDKs are serine/threonine kinases and part of the diverse protein kinase family. They are essential facilitators of life at the molecular level via their ubiquitous phosphorylation reactions. The majority of CDKs identified rely on binding partners called cyclins for their activation. In all there are 13 CDKs and 25 identified cyclins so far and though there is a high level of sequence and structural homology between them, knowledge of their function varies. A typical protein kinase consists of a small N-terminal domain formed of β-sheets and a large C-terminal domain (CTD) formed of α-

helices; between the terminals there is an ATP binding pocket and this pattern is also observed in CDKs. CDKs switch to an active conformation on binding to cyclin proteins which reveals enzymatic elements crucial to the catalytic function of their ATP-binding clefts. CDKs were named for this interaction with cyclin proteins which facilitates passage of cells through the G1, S, G2 and M phases of the cell division cycle (Meijer et al. 2003). Progression of the cell cycle depends more on the concentrations of endogenous CDK inhibitors (e.g. the WAF/CIP/KIP family) and cyclins than on the relatively stable CDK population. We commented earlier that CDK inhibitor drug action on neutrophils appears counter-intuitive but given the diversity of function that CDKs have been demonstrated to possess it is perhaps not surprising that another role for them has been found. Though initially identified as key components of the cell-cycle machinery they have subsequently been shown to play roles in cell differentiation, cell death (especially apoptosis), transcription and neuronal function. These alternative roles are mediated by different CDKs, an important factor in the drive to develop increasingly specific pharmacological CDK inhibitors (Knockaert et al. 2002).

Cyclin-dependent kinases 1 and 2

CDKs 1 and 2 have integral roles in the cell cycle, putative roles in transcriptional regulation and a controversial role in apoptosis (Golsteyn 2005). Traditionally, it has been believed that the CDK2 interaction with cyclin E facilitates G1/S transition though this role has recently been called into question by knockout mouse studies suggesting that the complex is dispensable for transition though it does have a role in centrosome duplication (Roberts et al. 2003). A second CDK2 complex, CDK2 –cyclin A, phosphorylates multiple substrates to inactivate G1 transcription factors and allow DNA replication. CDK 1 binds cyclin A at S/G2 transition but moves onto cyclin B to trigger G2/M transition and subsequently completes mitosis with the transition to anaphase (Golsteyn 2005). CDKs 1 and 2 have been implicated in induction and facilitation of the apoptotic process in some experimental scenarios whilst in others it is clear that their inhibition promotes apoptosis. CDK2 knockout in HeLa cells and CDK1 knockout in an FT210 cell-line resulted in a reduction in apoptosis whilst levels of CDK1-cyclin

complexes were noted to be elevated during apoptosis in HL-60 cells, YAC lymphoma cells and Jurkat cells. This observation had led some investigators to speculate that apoptosis might represent a form of 'failed mitosis' (Golsteyn 2005). Additionally, CDK inhibition with R-roscovitine appeared to prevent apoptosis in human hepatoma cell-lines and rat cerebellar neurons. In the latter model it was suggested that CDK1 phosphorylates BAD (a pro-apoptotic bcl-2 homologue) which facilitates its liberation from sequestration by 14-3-3 proteins. This allows BAD to translocate to the mitochondrial membrane where it mediates apoptosis (Konishi et al. 2002). This work is in complete contrast to experimental experience with CDKi in various cancer cell-lines where induction of apoptosis has been demonstrated (MacCallum et al. 2005). Several CDKs have roles in transcriptional regulation which is perhaps not surprising given the extensive degree of regulation that the transcription process receives from protein kinases. CDK2 has been implicated in cisplatin-related acute kidney injury through involvement in the release of E2F1 transcriptional machinery by phosphorylation of Retinoblastoma (Rb) protein which promotes transcription of pro-apoptotic genes (Obligado et al. 2007). Additionally, CDK2 inhibition has been implicated in the induction of apoptosis in diffuse large B-cell lymphoma where CDK2 was linked to transcription of Mcl-1 (an important survival protein). Apoptosis induced by CDK2 inhibition was associated with down-regulation of Mcl-1 and reduced phosphorylation of the enzyme responsible for Mcl-1 gene transcription, RNA polymerase II (RNA Pol II) (Faber et al. 2007). CDK1 mediated phosphorylation of the CTD of RNA Pol II has also been reported *in vitro* (Bregman et al. 2000). Despite a degree of controversy, the involvement of CDKs 1 and 2 in apoptosis and transcription (particularly of an important neutrophil survival protein) remains interesting when trying to understand the mechanism of action of CDK inhibitor drugs in the resolution of inflammation.

Cyclin-dependent kinase 5

CDK5 is perhaps most renowned as an important player in regulation of neuronal cyto-architecture and migration though evidence for its extra-neuronal abilities is increasing. So far, there is literature describing involvement in apoptosis, transcription,

differentiation and endocytosis. Interestingly, there is no evidence that it has any role to play in the cell-cycle and as it can function independently of cyclin proteins it is essentially mis-named. CDK5 has been implicated in the progression of apoptosis in mouse models of ovarian follicle degeneration, embryonic limb interdigital web apoptosis and digoxin-mediated prostate apoptosis (Rosales et al. 2006). CDK5 has also been shown to phosphorylate the important transcription factor STAT3 whose gene products include *c-fos* and *jun-b* as well as monocyte non-specific esterase. Of particular relevance to inflammation and apoptosis CDK5 complexed to p35 was expressed in Human promyelocytic leukemia cell-line (HL-60) induced to differentiate towards a monocytic phenotype. Specifically, this complex was shown to confer monocyte morphology-defining features such as CD14 and non-specific esterase suggesting a role for CDK5 in myeloid differentiation. Additionally, a key neutrophil function, GTP dependent secretion, was shown to depend on CDK5-p35 (Rosales et al. 2004). Numerous CDK5-p35 substrates were found within neutrophil granules whilst neutrophils treated with a CDK inhibitor drug lost GTP-dependent secretory function as well as CD63 and CD66b expression. Evidence of CDK5 involvement in transcriptional regulation, apoptosis and specific effects on inflammatory cells appears to be convincing (Dhavan et al. 2001).

Cyclin dependent kinases 4 and 6

CDKs 4 and 6 are required for progression through G1 phase and are activated by cyclin D in the presence of appropriate growth factors. As we have previously noted neutrophils are terminally-differentiated granulocytes. This irreversible cell-cycle arrest is mediated by withdrawal of CDK4 and 6 and up-regulation of p27kip1 (an endogenous CDK inhibitor). CDK4 and 6, like CDK2, phosphorylate retinoblastoma protein (Rb) to relieve elongation factor (EF)-2 transcription factors that initiate the switch to S-phase transcription (Klausen et al. 2004). CDK4 and 6 have been implicated in inflammatory events within the rheumatoid joint where they are said to modulate rheumatoid synovial fibroblast production of MMP-3 and monocyte chemotactic protein-1 (MCP-1) via Rb-dependent and -independent mechanisms (Nonomura et al. 2006). Importantly, cyclin

D/CDK4 complexes have been shown to activate the STAT transcription pathway independently of JAK in Drosophila and CDK4 and 6 have been shown to be important in leucocyte adhesion and migration (Liu et al. 2008;Silver et al. 2003). CDK4 and appear to have roles in inflammatory cell differentiation, adhesion and recruitment as well as inflammatory cytokine production and possibly inflammatory signalling. Targeting these CDKs would be facilitated by the current availability of specific pharmacological inhibitors of CDK4 and 6.

Cyclin dependent kinases 7 and 9

CDK7 is a CDK activating kinase responsible for enhancing the kinase activity of CDK1 and 2 by phosphorylation of the activation segment or T-loop. CDK7 associated with Cyclin H and menage a trois-1 (MAT1) is responsible for initiation of transcription by the holoenzyme RNA Pol II and mediates this effect by phosphorylation of the CTD. This is a function it shares with CDK9 which, when associated with cyclin T, is responsible for transcriptional elongation by RNA Pol II (Oelgeschlager 2002). CDK7 and 9 have been implicated in transcription during early infection by CMV and are known to be important in activation of HIV1 transcription (Fisher 2005;Kapasi et al. 2008). Perhaps most interestingly, CDKs 7 and 9 with their respective binding partners have been shown to play an integral role in the aberrant survival of multiple myeloma cells. This effect is thought to be mediated by RNAPol II transcription of the survival protein Mcl-1. R-roscovitine, a CDKi which has specificity for CDK 1,2,5,7 and 9, was able to promote apoptosis in these cells which was associated with down-regulation of Mcl-1 (MacCallum et al. 2005). Additionally CDK9 has also been shown to bind TNF receptor associated factor-2 (TRAF2) a protein that is of known importance in the activation of NF-κB mediated by TNF-α (MacLachlan et al. 1998;Wang et al. 2008). Regulation of transcription of Mcl-1 (a key neutrophil survival protein) and involvement in pivotal inflammatory signalling via NF-κB are functions of CDKs 7 and 9 that might be applicable to the regulation of neutrophil apoptosis in inflammatory resolution.

1.4.2 Cyclin-dependent kinases in neutrophil granulocytes

As neutrophils do not undergo cell division and can progress no further in the cell-cycle (they are thought to remain in G_o phase) CDKs had not been thought relevant to their biology. There is, therefore, a paucity of information about CDKs, their interactions and potential roles in these cells. CDK1 and CDK2 proteins had been identified in neutrophils but there was little indication of their function. No change in CDK1 or CDK2 protein expression was found in a variety of neutrophil populations including fresh human neutrophils, aged human neutrophils, survival factor treated neutrophils at various time-points and CDK inhibitor drug treated neutrophils (Rossi et al. 2006). This is perhaps not a surprising finding as levels of CDKs 1 and 2 are relatively constant throughout the cell cycle and it is changes in expression of their binding partner cyclins and endogenous CDK inhibitors that modulate effects on cell-cycle progression (Knockaert et al. 2002). It cannot be assumed therefore, that these CDKs are not important in the induction of apoptosis even though they are not targets for degradation. As previously discussed CDK1 and CDK2 can bind to various cyclin subtypes, whereas CDK5 binds non-cyclin partners including p35 and p39 regulatory proteins. In addition, CDK5 is expressed in human neutrophils as evidenced by detection of CDK5 mRNA and protein (Rosales et al. 2004). Evidence of CDK1, CDK2 and CDK5 activity in neutrophils is scarce but available. Our group have demonstrated a prompt reduction in CDK1 activity during induction of apoptosis by the activating Fas antibody CH11. The CDK-binding partners and regulatory proteins in neutrophils remain to be identified (Rossi et al. 2006). It is known that despite the terminally differentiated status of neutrophils they are capable of phenotypic alteration. Indeed, it has been postulated following work with the 'MacGreen' mouse that mouse neutrophils sufficiently stimulated with CSF-1 *in vitro* can transdifferentiate into dendritic-type cells (Sasmono et al. 2007). This has implications for the role of neutrophils in the resolution of inflammation as it may significantly extend their capability to positively contribute. Another phenotypic switch from generation of pro-inflammatory mediators to anti-inflammatory and/or pro-resolving mediators such as lipoxins and resolvins has also

been identified (Serhan 2007). This raises the possibility that a transcriptional effect might be mediated by up or down-regulation of protein kinases such as the CDKs. More information is required about CDKs, cyclins, endogenous CDK inhibitors and their function in inflammatory cells.

1.4.3 Cyclin-dependent kinase inhibitor drugs in inflammation

The research discussed above led our group to investigate a role for CDK inhibitor drugs in inflammation. Our research paradigm has been that inflammation should be driven down resolution pathways by the induction of apoptosis in granulocytes followed by their effective clearance by professional phagocytes such as macrophages (Leitch et al. 2008). Rossi et al initially examined the *in vitro* effects of R-roscovitine, NG-75 and hymenialdisine on human neutrophils at different time-points and drug concentrations (Rossi et al. 2006). These CDK inhibitor drugs are structurally diverse but their uniform effect was to promote neutrophil apoptosis in a time and concentration dependent manner as evidenced by annexin-V binding and morphological assessment. Neutrophils treated with R-roscovitine and the caspase-inhibitor zVAD-fmk failed to enter apoptosis, suggesting that R-roscovitine was acting in a caspase-dependent manner. This was evident at 4 hours post-incubation when caspase-3 cleavage was already detectable. The most intriguing *in vitro* result was the ability of the CDK inhibitor drugs to overcome diverse survival factors including db-cAMP, GM-CSF and LPS. These survival factors utilise the major inflammatory signalling pathways: PI3K, NF-κB, JAK/STAT and MAPK, to augment neutrophil survival. Given that the major hurdle to development of anti-inflammatory agents is the redundancy conferred by these same signalling pathways this result underlined the potential of CDK inhibitor drug therapy for treatment of inflammatory disease. Inflammation research demands *in vivo* experimentation as it is impossible to recreate the inflammatory milieu *in vitro* and hence to predict the efficacious translation of an agent that has been successful *in vitro*. R-roscovitine was investigated in three mouse models of neutrophil dominant inflammation including: carageenan-induced pleurisy, bleomycin lung injury and passively induced arthritis. In

the carageenan pleurisy model 100mg/kg of R-roscovitine administered intraperitoneally reduced an established inflammatory infiltrate to near basal levels consistent with those found in an untreated mouse pleural cavity. There was a reduction in populations of inflammatory cells (including neutrophils, monocytes and macrophages), oedema formation and pro-inflammatory cytokines. This effect was reversed *in vivo* by administration of the caspase inhibitor zVAD-fmk. In mice with established bleomycin-lung injury there was a reduction in BAL neutrophil numbers assessed after three days, a reduction in histopathological lung inflammation after 7 days and an effect on bleomycin-induced lethality. Finally, in mice with established passively-induced arthritis there was an improvement in clinical scores of arthritis following R-roscovitine administration (Rossi et al. 2006). These *in vivo* findings suggest encouraging pleiotropic effects of CDK inhibitor drugs on granulocyte recruitment, survival and removal. Pleiotropic effects of CDK inhibitors are supported by work from Liu et al who have shown that CDK4 is important in leucocyte recruitment and adhesion. They studied CDK4 -/- knockout mice with bleomycin lung injury and utilised siRNA to CDK4 and CDK inhibitor drugs to show that CDK4 inhibition inhibited leucocyte recruitment in the mouse model and leucocyte adhesion in endothelial cell (EC) matrix models (Liu et al. 2008). In addition Sekine et al have now demonstrated positive effects of flavopiridol and a specific CDK4, 6 inhibitor on animal models of rheumatoid arthritis (Sekine et al. 2008). Their findings suggest lymphocyte independent effects of CDK inhibitors (including down-regulation of fibroblast proliferation and growth) are responsible for improvement in joint histology and clinical arthritis scores in various mouse models. Findings in inflammatory joint and lung disease models are mirrored in kidney disease models where the CDK inhibitor drug R-roscovitine has entered phase 1b clinical trials for inflammatory kidney disease. Glomerulonephritides are characterised by inflammation and progressive, scarring destruction of key functional kidney units leading to renal dysfunction and failure. Pre-clinically, CDK inhibitor drugs have been shown to protect renal tubular epithelium from enhanced apoptosis whilst inhibiting the abnormal proliferation of tubular epithelial and mesangial cells. *In vitro* and *in vivo* work has demonstrated that R-roscovitine can restore normal kidney function in animal

models of IgA-mediated glomerulonephritis, crescentic glomerulonephritis, lupus nephritis and collapsing glomerulonephropathy (Gherardi et al. 2004;Milovanceva-Popovska et al. 2005;Obligado et al. 2007;Zoja et al. 2007). The work with NZBxNZW mice affected by early or established proliferative lupus nephritis is particularly interesting as whilst leucocyte driven inflammation was shown to be reduced there was also evidence of a direct effect of CDK inhibitor drugs against autoimmune T- and B-lymphocyte responses. It is perhaps less surprising that CDK inhibitors should work in this setting given the proliferative potential/state of differentiation of lymphocytes but nonetheless the possibility of pleiotropic action against autoimmune inflammation is an exciting one.

Further evidence for the utility of CDK inhibitors has been provided by research into the properties of endogenous CDK inhibitors. The physiological CDK inhibitor p21 (WAF1, SD1, Cip1), a specific inhibitor of CDK2, 4 and 6 has been shown to negatively regulate macrophage activation by reducing TNF-α and IL-1βproduction in response to LPS. Additionally, p21-/- mice have an increased susceptibility to LPS-induced shock which is associated with elevated levels of IL-1β (Lloberas et al. 2009;Scatizzi et al. 2009). In an inflammatory lung disease model, p21 was over-expressed in the lungs of mice subjected to bleomycin injury by an intra-tracheal adenoviral transfer method (Inoshima et al. 2004). p21 expression in lung epithelial cells led to a reduction in lung inflammation, preservation of epithelial cells and reduced fibrosis. In rheumatoid arthritis patients, p21 gene transfer was shown to down-regulate expression of inflammatory mediators and tissue-degrading proteinases such as: IL-6, -8, type I IL-1 receptor, MCP-1, MIP-3α, cathepsins B and K, and MMPs -1 and -3 (Nonomura et al. 2003).

In summary, CDK inhibitor drugs promote neutrophil apoptosis *in vitro* even in the presence of powerful survival factors and promotes resolution of inflammation, *in vivo*, in various animal models of neutrophil-dominant inflammation. Neutrophil apoptosis has been shown to be central to the resolution of inflammation by caspase inhibition which reversed the beneficial impact of CDK inhibitors. In addition the anti-proliferative and

anti-apoptotic effects of CDK inhibitors protect epithelia against inflammatory insult. CDK inhibitor drugs also prevent lymphocyte proliferation and pro-inflammatory signalling indicating potential effects against chronic and autoimmune, inflammatory disease. An understanding of the mechanism of action by which these powerful anti-inflammatory effects are achieved may allow optimisation of CDK inhibitor drugs and suggest further targets for pharmacological intervention.

1.4.4 Cyclin-dependent kinase inhibition

Cyclin-dependent kinase inhibitor drugs such as R-roscovitine have emerged as potential, anti-inflammatory agents that augment neutrophil apoptosis and suppress lymphocyte proliferation and secretory function (Obligado et al. 2007;Rossi et al. 2006). This former finding seems counter-intuitive because the neutrophil is a terminally-differentiated cell and its cell-cycle machinery ought to be effectively vestigial. Further research is required before we develop a functional understanding of the mechanism of action of CDK inhibitor drugs in inflammation but already some tantalising clues are emerging. This section will focus on the CDKs, their inhibitors, the role of CDK inhibitors in inflammation and areas for further research. It is anticipated that CDK inhibitor drugs will provide the basis for the development of novel therapeutic agents that drive resolution of inflammation and counter inflammatory disease.

Pharmacological CDK inhibitors

Pioneering work on the cell-cycle in starfish oocytes in the 1990s, driven by the realisation that anti-mitotic agents would make effective anti-cancer drugs, led to the discovery of CDK inhibitors (Meijer et al. 2003). The widely available, non-specific kinase inhibitors staurosporine, 6 D-MAP and isopentanyladine were found to potently inhibit CDK1/cyclin B but were too non-specific in action to yield useful information about the functional impact. A laborious screening process subsequently uncovered olomoucine, a purine analogue that showed enhanced specificity for CDKs but some

action against MAPKs. This compound was shown to mediate its inhibitory effect by competing with ATP for a binding site on CDK1. Further structural analysis of this ATP-binding site interaction identified the 2,6,9 trisubstituted purine family as likely candidates for effective CDK inhibition. This was the development that brought R-roscovitine and Purvalanol B into the frame and led to the identification of a family of approximately 50 CDK inhibitors (Table 1) (Meijer et al. 2003).

Table 1: IC50 values for selected CDK inhibitor drugs (Bettayeb et al. 2007;Knockaert et al. 2002;Krystof et al. 2002;Obligado et al. 2007)

	IC50 values for kinase inhibited by CDK inhibitor (μM)								
	CDK1/ cyclin b	CDK2/ cyclin E	CDK 4	CDK 5	CDK 7	CDK 9	GSK	ERK 1	ERK 2
R-roscovitine	0.45, 2.7	0.13, 0.7	14.2, 14.7, >100	0.16	0.49	0.74, 0.78	130	34	14
Olomoucine	7	7	>1000	3			100	30	50
Olomoucine II	7.6	0.1	19.8		0.45	0.06	>100		32
Purvalanol B	0.006	0.009	>10	0.006			>10	3.33	
Flavopiridol	0.06, 0.4	0.15	0.4	0.17	0.3	0.006	0.45		
Aminopurvalanol	0.033	0.028		0.02				12	2.4
Hymenialdisine	0.022	0.04	0.6	0.028			0.01	0.47	2
Fascaplysin	>100	>50	0.35	20					
OL567	0.23								
H717	0.23	0.05							
NU2058	5	12							
NU6027	2.5	1.3							
Staurosporine	0.006	0.007	<10					0.02	0.02
PD183812	>40	0.17	0.008						
Meriolin 1	0.78	0.09		0.51		0.026	0.63		
SU 9516	0.04		0.2						
DRB					20	3			
Alsterpaullone	0.035	0.2	>10	0.04			0.004	22	4.5

CDK inhibitor drugs are universally (to date) flat, hydrophobic heterocycles of low molecular weight that mediate binding to the ATP pocket via hydrophobic interactions and hydrogen bonds. Surprisingly, despite this uniformity, it is possible to categorise the CDK inhibitors on the basis of their relative specificity for CDKs: 1. Non-specific (Flavopiridol), 2. CDK 1,2,5,7-specific (Olomoucine, R-roscovitine and Purvalanol B), 3. CDK 4,6-specific (fascaplysin, PD0183812). It is clear that any discussion of CDK inhibitor specificity for individual CDKs can only be couched in relative terms. Additionally, there are some non CDK targets of these inhibitors identified on random screening which include ERK, GSK3 and pyridoxal kinase (Knockaert et al. 2002). Given the range of activities performed by CDKs on a variety of vital cell processes a lack of incisive specificity attributable to an inhibitor makes it extremely difficult to assign outcomes to specific actions on defined targets. The basis for this work is that CDK inhibitor drugs have proven efficacy in models of inflammation but the reality is that these effects may not relate to any action on CDKs themselves. An obvious point of interest then, is the inhibitory effect of certain CDK inhibitor drugs on the ERK signalling pathway. Recent work has shown that specific ERK-inhibitors can enhance the resolution of carageenan-induced pleurisy in rats, an accepted model of acute inflammation (Sawatzky et al. 2006). However, it seems likely that the inhibitory effect of CDK inhibitors on the ERK pathway would not account for the anti-inflammatory actions of R-roscovitine. In addition, the biological effect of CDK inhibitors and ERK inhibitors on neutrophil apoptosis differ markedly. For example: the ERK inhbitor PD98059 does not induce neutrophil apoptosis *per se* whereas CDK inhibitor drugs do; R-roscovitine reverses LPS, GM-CSF and dibutyryl (db)-cAMP mediated prolongation of neutrophil survival whereas PD98059, only reverses LPS and GM-CSF survival; finally, PD98059 completely inhibits LPS induced phospho-ERK expression in neutrophils whereas R-roscovitine does not (Leitch et al. 2010b). Regardless of the difficulties associated with non-specificity the ability of CDK inhibitor drugs to selectively induce apoptosis in actively proliferating cells has meant that research into their use as anti-cancer agents has progressed rapidly.

CDK inhibitor drugs including R-roscovitine, flavopiridol and SU9516 have anti-cancer actions and R-roscovitine alone is known to reduce the proliferation index of 19 distinct cancer cell-lines *in vitro* (McClue et al. 2002). The mechanism of action of CDK inhibitors in cancer cell-lines whilst not directly applicable to inflammatory cells is of great interest to those interested in targeting CDKs to resolve inflammation. CDK inhibitors were an exciting therapeutic prospect because CDKs interact with the Rb family widely regarded as the master-switch of the cell cycle. CDKs are overabundant in some cancers resulting in excessive phosphorylation and functional inactivation of Rb, allowing unregulated proliferation (Johnson et al. 1994). However, it was noted that R-roscovitine was capable not only of inducing cell cycle arrest, but of active promotion of apoptosis. The induction of apoptosis by R-roscovitine in myeloma cell-lines has been reported to be promoted by CDK modulation of RNA Pol II mediated transcription of Mcl-1. Additionally, down-regulation of XIAP, another Bcl-2 homologous survival protein, was observed in chronic lymphatic leukaemia cells and apoptosis in both cell-lines was found to be caspase-dependent (Hahntow et al. 2004;MacCallum et al. 2005). A recent paper has shown inhibition of the well known inflammatory transcription factor, NF-κB by R-roscovitine in A549, 293, H1299 and ARN8 cancer cell lines (Dey et al. 2008). It is tempting to directly extrapolate from these findings to inflammation studies but given the sometimes, directly contrasting effects of pharmacological agents in different cell-types (for example the anti-apoptotic effects of CDK-inhibition in cerebellar neuronal cells) we must await further definitive studies. The availability of this mechanistic information and encouraging safety profiles in animal studies have allowed progression to clinical trials of CDK inhibitor drugs in conditions such as B-cell malignancy, non-small cell lung cancer and breast cancer. The side-effect profile has been promising but includes tolerable and limited gastrointestinal disturbance, skin rash, reversible transaminitis and hypokalaemia (Senderowicz 2003). This transition to human studies makes an elucidation of the mechanism of action of CDK inhibitor drugs in inflammation imperative, as trials of these drugs in inflammatory disease are likely to be on the near horizon.

1.5. Modelling inflammatory lung disease *in vivo*

There are many methods available for the production of relevant animal models of inflammatory disease. These include: direct chemical/other injury, constitutive and inducible transgenics, viral-vector delivery of relevant genes, adoptive cell transfer and direct infection strategies. The advantage of the animal model is that it reproduces the complexity of the wider immune/inflammatory response on an organ or systems-wide basis as opposed to cell-line strategies which can only examine individual cellular responses. Clearly, reliable animal models are central to the development of efficacious and safe pharmaceutical agents that drive granulocyte apoptosis and clearance to enhance resolution of inflammation. The animal model examined in this thesis is the murine bleomycin acute lung injury model.

1.5.1 Bleomycin lung injury

Murine bleomycin lung injury is one of the most widely used and well characterised animal models of lung disease described. The drug bleomycin is an antibiotic that can be isolated from *streptomyces verticillatus* and has been used as an antineoplastic agent for a number of cancers including Hodgkin's lymphoma and testicular cancer. It was in this setting that toxic lung fibrosis was first encountered as an unwanted side-effect. Bleomycin causes damage by inducing DNA strand brakes and causing oxidative damage. It induces dose-dependent lung injury and fibrosis in a range of animals and it can be administered intravenously (*i.v.),* intra-tracheally *(i.t.),* subcutaneously *(s/c)* or intraperitoneally *(i.p.)*. There are advantages to each method of administration but the usual outcome is acute lung injury consisting of necrotic loss of alveolar epithelium, hyaline membrane formation, alveolar consolidation (neutrophil/lymphocyte influx) and protein leak followed by a fibrotic reparative phase of collagen deposition (Moeller et al. 2008;Moore et al. 2008).

At 1-7 days following bleomycin administration the major histopathological events are epithelial cell damage and loss and an intense mixed infiltrate of neutrophils, activated macrophages and lymphocytes within the alveolar spaces and parenchyma. The inflammatory cytokines TNF-α, IL-6, IL-8 and GM-CSF are said to predominate in the acute phase. During days 7-14 the inflammatory infiltrate begins to clear and fibroblast proliferation with extra-cellular matrix deposition predominate. At day 14 the first signs of fibrosis are evident and this persists and progresses to day 28. TGF-β and other genes regulated by it are the major drivers of the fibrotic phase. Beyond this point lung fibrosis begins to resolve spontaneously, a feature that is the major weakness of the murine model in comparison to the relentlessly progressive human disease. The major strengths of this model are its reproducibility and similarity to human disease pathology especially in the production of predominantly bronchiolocentric fibrotic changes and acute alveolar and interstitial inflammation leading to epithelial cell death and basement membrane damage (Moeller et al. 2008;Moore et al. 2008).

In this thesis the acute lung injury phase of the bleomycin model is investigated because of the specific focus on neutrophils and neutrophil apoptosis in the resolution of inflammation.

1.6 Summary of Introduction and Aims

Inflammation is the main cause or a major contributor to the pathogenesis of a number of lung diseases. Many of the damaging sequelae of inflammation are attributable to neutrophil granulocytes that are either inappropriately activated, unnecessarily persistent or insufficiently regulated. A strategy for the resolution of inflammation may involve the promotion of neutrophil granulocyte apoptosis provided that professional phagocytes are able to clear the increased apoptotic cell burden. Neutrophils are critically dependent on the bcl-2 homologue survival protein Mcl-1 which is known to be rapidly turned over within the cell. CDKs are key proteins involved in the regulation of the cell cycle but also have important roles in transcription and apoptosis. CDK inhibitor drugs can drive neutrophil apoptosis and resolution of inflammation. This thesis will aim to investigate:

1. The mechanism by which CDK inhibitor drugs induce neutrophil apoptosis
2. The molecular events involved in apoptosis driven by CDK inhibitor drugs
3. The relevance/importance of CDKs as targets of CDK inhibitor drugs in the induction of neutrophil apoptosis
4. The possibility of producing molecular biology tools/agents to reliably and specifically knockdown key targets of CDK inhibitor drugs in neutrophil granulocyte cells
5. The mechanisms by which inflammation is resolved by CDK inhibitor drugs in the bleomycin lung injury model

1.7 Hypotheses

1. **CDK inhibitor drugs drive neutrophil apoptosis by the intrinsic (mitochondrial) pathway**
2. **Mcl-1 is required for neutrophil survival**
3. **Neutrophils have transcriptional machinery and functional CDKs**
4. **CDK inhibitor drugs target CDKs 7 and 9 to down-regulate transcription of the key survival protein Mcl-1**
5. **Neutrophils are dependent on their transcriptional capacity for survival**
6. **CDK inhibitor drugs have specific effects on neutrophil transcriptional capacity**

2. Materials and Methods

2.1 Materials

TRIzol® Reagent (Gibco)

Diethyl Pyrocarbonate (DEPC) (Sigma)

NDSB-201 (3-(1-Pyridino)-1-propane sulfonate)

18S competimers (Applied Biosystems)

2-(R)-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (*R*-Roscovitine; Calbiochem, UK)

2-Val-Ala-DL-Asp (OMe) Flrylmethoketone (Z-Vad-fmk; Bachem, Switzerland)

5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB; Sigma)

Annexin V Fluos (Roche)

BioMax MS-1 light–sensitive film (Kodak)

Bleomycin (Apollo Scientific)

Calcium chloride (Sigma)

CDK4 inhibitor (2-Bromo-12,13-dihydro-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7(6H)-dione, Calbiochem)

Cell culture medium and Trypsin/EDTA (PAA Laboratories Ltd, UK)

CellTracker™ Green (Molecular Probes, Invitrogen, UK)

DAPI (Invitrogen)

Dexamethasone, LPS (E Coli) (Sigma)

Dextran (Pharmacosmos)

Diff-Quik™ (Gamidor Ltd., Abingdon, GB).

ECL reagents (GE Healthcare)

Fetal Calf Serum (Biosera, UK)

FITC (Sigma)

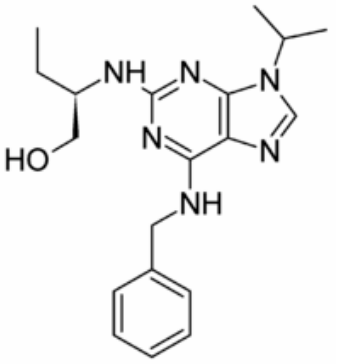
Gliotoxin (Calbiochem)

Mcl-1 primer pair (R&D)

MG-132 (Calbiochem)

Mitocapture (Biotium)

HEPES (Sigma)



R-roscovitine

PCR Master-mix (Promega)

Percoll^{TM} (GE Healthcare, UK)

PI (Sigma)

Precision Precast gels (Pierce)

PVDF (Immobilon-P, Millipore)

Recombinant human GM-CSF (R&D Systems)

Recombinant human TNF-α (R & D Systems, Abingdon, Oxon, UK)

Mcl-1 primers (eurofins): Mcl-1FOR 5'-GCGTAACAAACTGGGGCAG-3', Mcl-1REV 5'-GCAAAAGCCAGCAGCACATT-3'

XIAP primers (eurofins): XIAPforward 5'-GGAACCTTGTGATCGTGCCT-3'

XIAPreverse 5'-AATCAGTTAGCCCTCCTCCA-3'

CDK7 primers (eurofins):

CDK7DNFOR1 5'GAATTCGATGGCTCTGGACGTGAAGTC TCG-3' HPLC+

CDK7DNREV1 5'-GCGGCCGCTTAAAAAATTAGTTTCTTGGGCAA-3' HPLC+

CDK7DNFOR2 5'-GGCTTATTCTTATTTAATCCATGTG-3' HPS

CDK7DNREV2 5'-GACAAGGCTAATATTAGATTTATGT-3' HPS

CDK9 primers (eurofins):

CDK9FOR1 5'-GAATTCTATGGCAAAGCAGTACGACTCGGTG-3' HPLC+

CDK9REV1 5'-GCGGCCGCTCAGAAGACGCGCTCAAACTCCGT-3' HPLC+

CDK9FOR2 5'-CCTGCCCAGCGCATCGACAGCGATG-3' HPS

CDK9REV2 5'-CTTCTTCAGAGCCACCTTCTGGCC-3' HPS

T7 primer (eurofins): 5'-TAATACGACTCACTATAGGG-3'

2.2 Buffers

4x Sample Buffer: 2.4ml 1 M Tris pH 6.8, 0.8 g SDS stock, 4ml 100% glycerol, 0.01% bromophenol blue, 1ml ß-mercaptoethanol, 2.8ml water.

Annexin-V binding buffer (HBSS (w/o cations) + 5mM $CaCl_2$).

IMDM, HBSS, PBS, PBS 10x (Life Technologies, Paisley, United Kingdom).

Whole cell lysis buffer (standard): 0.5ml aliquot of 1:50 dilution of Sigma Protease cocktail P8340 in 1xTBS plus 0.3ml 1xTBS, 1.5mM EDTA, 10mM KCl, 0.5mM dithiothreitol, 1mg/ml aprotinin, leupeptin, and pepstatin A, 1mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1mM sodium orthovanadate, 0.5mM benzamidine, and 2 mM levamisole.

TBE: 0.45M Tris, 0.45M Boric acid, 10mM EDTA, pH 8.3.

Running Buffer: 100mM Tris, 100mM HEPES, 3mM SDS, pH 8.

Transfer Buffer: 25mM Tris, Glycine 192mM, 10% methanol.

TBS: 50mM Tris, 150mM NaCl, pH 7.4.

Blocking solution: TBS/Tween (0.1%)/Dried milk (Marvel) 5%.

Cytoplasmic Lysis Buffer: AEBSF 0.5mM, HEPES 50mM, KCl 50mM, $MgCl_2$ 2mM, EDTA 0.1mM, Aprotinin 10μg/ml, Levamisole 2mM, Leupeptin 10μg/ml, Sodium orthovanadate 1mM, Benzamidine 0.5mM, β-Glycerophosphate 10mM, Pepstatin A 10μg/ml, Phenanthroline 1mM, PMSF 1mM.

Nuclear Lysis Buffer: AEBSF 0.5mM, HEPES 50mM, KCl 50mM, NaCl 300mM, Glycerol 10%, EDTA 0.1mM, Aprotinin 10μg/ml, Levamisole 2mM, Leupeptin, 10μg/ml, Sodium orthovanadate 1mM, Benzamidine 0.5mM, β-Glycerophosphate 10mM, Pepstatin A 10μg/ml, Phenanthroline 1mM, PMSF 1mM.

Resuspension buffer: 25mm NaH_2PO_4.

Solubilisation buffer: 6M Guanidine HCl, 25mm NaH_2PO_4, 0.5M NaCl, 1mM β-mercaptoethanol.

Refolding buffer 1: 25mM NaH_2PO_4, 0.5M NaCl, 1mM β-mercaptoethanol.

Refolding buffer 2: 50mM HEPES pH 7.5, 0.2M NaCl, 1mM DTT; 1mM reduced glutathione; 10% Glycerol; 0.5M Guanidine HCl; 1mM EDTA. (DTT and glutathione added fresh).

Refolding buffer 3: 50mM HEPES pH 7.5, 0.2M NaCl, 1mM DTT, 1mM reduced glutathione 10% Glycerol, 0.5M NDSB-201, 1mM EDTA. (DTT and glutathione added fresh).

Elution buffer 1: 25mM NaH_2PO_4, 0.5M NaCl, 1mM β-mercaptoethanol, 250mM imidazole.

Elution buffer 2: 6M Guanidine HCl, 25mm NaH_2PO_4, 0.5M NaCl, 1mM β-mercaptoethanol, 250mM imidazole.

Elution buffer 3: 50mM HEPES pH 7.5, 0.2M NaCl, 1mM DTT, 1mM reduced glutathione, 10% Glycerol, 0.5M NDSB-201, 1mM EDTA. (DTT and glutathione added fresh) 250mM imidazole.

2.3 Antibodies

Anti-Mcl-1(Santa Cruz)
Anti-XIAP (R&D)
Anti-Bid (Santa Cruz)
Anti-Bax (Santa Cruz)
Anti-Bim (Cell Signalling)

Anti- IκBα (Abcam)

Anti-β-actin (Sigma-Aldrich)

Goat anti-mouse/-rabbit and rabbit anti-goat horseradish peroxidase (HRP)–conjugated (Dako)

Anti-RNA polymerase II (PS 2, PS 5, Total) (Covance)

Anti-CDK 2,5,7,9 (Santa Cruz)

Anti-cyclin T1, H, MAT1 (HRP-conjugated, Santa Cruz)

Alexa (Goat anti-Mouse) 647 Fab fragments (Invitrogen)

Alexa (Goat anti-Rabbit) 647 Fab fragments (Invitrogen

2.4 Methods

2.4.1 Cell Isolation and Culture

Polymorphonuclear leukocytes were isolated from peripheral venous blood of healthy human donors. Approximately 40-160ml of blood was isolated on any one occasion and collected in 50ml polypropylene tubes containing sterile sodium citrate (to final conc. 0.38%). After centrifugation of citrated whole blood at 300*g* for 20min and removal of platelet-rich plasma, leukocytes were separated from erythrocytes by dextran sedimentation over 25-30min using pre-warmed 0.6% (wt/vol) dextran T500. The upper leukocyte-rich layer was carefully aspirated and the volume adjusted to 50ml using pre-warmed 0.9% saline. Leukocytes were subsequently pelleted by centrifugation at 350g for 6min. Polymorphonuclear leukocytes were then separated from mononuclear leukocytes using discontinuous isotonic Percoll (a 9:1 ratio of percoll:10xPBS) gradients (81%, 70% 55% Percoll in 1xPBS without cations). Gradients were prepared by layering 3ml of 70% isotonic percoll on 3ml of 81% isotonic percoll in a 15ml polypropylene tube before layering leucocytes re-suspended in 3ml 55% percoll on top. Gradients were centrifuged at 720g for 20min at which point mononuclear cells were found at the upper 55:70% interface while polymorphonuclear leucocytes were at the 70:81% interface. Cell yield was estimated with a bright-line haemocytometer. Leukocytes underwent a

further two wash steps in pre-warmed 1xPBS without cations. Polymorphonuclear leukocytes were at least 98% neutrophils using morphologic criteria and cell viability was assessed by trypan blue exclusion. Cells were re-suspended at $5x10^6$/ml in IMDM plus 50U/ml penicillin and 50U/ml streptomycin plus 10% autologous serum and treated in 2ml eppendorf tubes at 37^oC on a shaking heat block or cultured in flat-bottomed flexible well plates in a humidified, 5% CO_2 atmosphere at 37 º C.

2.4.2 Assessment of granulocyte apoptosis by microscopy and flow cytometry

Neutrophil apoptosis was assessed morphologically by cyto-centrifuging 100µl of $5x10^6$ cells/ml at 300rpm for 3min. Cells were air-dried then fixed for 1min in methanol and stained with Diff-Quik^{TM}. Morphology was assessed at 100x objective on an oil-immersion microscope. Apoptotic cells were defined as those containing one or more darkly stained pyknotic nuclei (500 cells were counted over 5 fields of view). Additionally, flow cytometric assessment of annexin-V binding and propidium iodide (PI) staining were used to assess apoptosis and necrosis respectively. Annexin-V was diluted 1/500 in binding buffer (HBSS + Ca^{2+}) and 280µl added to 20µl of cells ($5x10^6$cells/ml). Samples were then incubated on ice at 4°C for 10min. Immediately prior to processing on the Coulter FACSCaliber flow cytometer (Beckman Coulter, California, USA), 1µl of PI (1mg/ml) per sample was added. Data were captured with the use of cellquest software (Becton Dickinson) with FL1 designated as Annexin-V positivity while FL2 reflected PI positivity. Measurements were made as percentages of total gated neutrophils.

2.4.3 Standard cell lysis protocol

Cells were pelleted at 10000g for 30s then re-suspended in 180µl of whole cell lysis buffer they were then incubated on ice for 10min. Following this 10µl of 10% NP-40 lysis buffer was added and samples quickly vortexed before incubating on ice for a

further 10min. The samples were then centrifuged at 10000g, 4°C for 20min after which supernatants were removed, 4x sample buffer added and samples boiled on a 95°C heat block for 5min and stored at -20°C/-80°C.

2.4.4 Western Blotting

Cells at a concentration of $5x10^6$/ml per condition were incubated at 37°C on a shaking heat block. For times and reagent concentrations please refer to figure legends. Lysates, prepared as described above, and run on 4, 10, or 12% SDS gel (either precast gels or prepared to above recipe) and transferred onto PVDF. Membranes were blocked for 1h in 5% (wt/vol) dried milk/TBS/0.1% Tween-20 or 5% (wt/vol) BSA/TBS/0.1% Tween-20 prior to overnight incubation at 4ºC (or 1.5h at room temperature for β-actin and HRP conjugated primary antibodies) with primary antibodies diluted as indicated above. Following 3x 5 min washes in TBS/0.1% Tween-20 blots were incubated with the appropriate HRP-conjugated secondary antibody diluted 1:2500 for 1h at room temperature (if required) prior to incubation with ECL reagents, exposure to light–sensitive film, and processing through an x-ray developer (X-Ograph Imaging Systems).

2.4.5 RNA isolation and reverse transcriptase-polymerase chain reaction (PCR) (semi-quantitative)

Total neutrophil RNA was isolated with the sole use of a Nucleospin RNA II kit (Macherey-Nagel) as per manufacturer's instructions apart from for the illumina gene chip experiment where a dual method with a trizol™ extraction stage was performed. 2µg of RNA was made up to a final volume of 25µl with DEPC-ddH_2O. Reaction mix for each sample prepared as follows: 10µl 5x M-MLV reaction buffer, 5µl 100mM DTT, 5µl 100µg/ml oligo(dT) primer, 4µl 10mM dNTPs, 0.5µl Rnasin, 0.5µl M-MLV reverse transcriptase. 25µl of this mix added to each 2µg RNA sample prior to incubation for 90 min at 37°C to allow cDNA synthesis. Reaction stopped by incubating at 90°C for 10min. 2µL Primers and 5µl cDNA added to 25µl Master-mix and volume

made up to 50µl with DPEC H_2O. Tubes placed in thermal-cycler with following program: 94°C for 4min then: 94° C for 45s, 55° C for 45s, 72° C for 45s x 30 - 35 cycles, 72° C for 10min. Products separated by agarose gel electrophoresis on 1-2% (in TBE) gels (using GelRed nucleic-acid detection dye/ethidium bromide) in TBE prior to visualisation and image capture with a UV-light camera.

2.4.6 Immunohistochemistry and confocal microscopy

Freshly isolated neutrophils at $2.5x10^6$/ml or incubated with treatments in 2ml eppendorfs on 37°C shaking heat block for indicated time-points were pelleted by centrifugation at 300g for 4min prior to resuspension and transfer to coverslips. They were then fixed for 20min in 3% paraformaldehyde (PFA). After washing and quenching with 50mM glycine, cells were re-suspended in 50µl of 10% goat serum (as secondary antibodies were goat) and blocked for 1h. Supernatant was removed and coverslips were gently placed onto 100µl of indicated primary antibody diluted as indicated for 1h. Following washing, the cells were incubated with 50µl of appropriate fluorescent-conjugated secondary antibody as well as nuclear stain (DAPI/PI, 2µl in 1ml) for 1h before further washing in PBS and finally ddH_2O. Coverslips were transferred to slides and mounted with mowiol and nail varnish prior to visualization on confocal microscope (Zeiss LSM510meta/Leica SP5). All experiments were performed with controls in the form of unlabelled cells, cells labelled with primary antibody only and cells labelled with secondary antibody only.

2.4.7 Neutrophil/cytoplasmic differential lysis

Neutrophils incubated in 2ml Eppendorf tubes on shaking heat block (37°C) and pelleted by centrifugation at 3000g for 60s and re-suspended in 100µl of cytoplasmic lysis buffer. Sample incubated on ice for 10min then 10µl of NP-40 added, briefly vortexed and centrifuged for 10min at 4300g. Supernatant removed, added to appropriate volume of sample buffer and boiled for 2min at 95 °C. Remaining cell pellet re-suspended in

50µl of nuclear lysis buffer and placed at 4 ^{o}C plus constant shaking for 20min to solubilise nuclear proteins. Samples then centrifuged at 23100g for 10min and supernatants added to appropriate volume sample buffer before boiling at 95 ^{o}C for 2min.

2.4.8 Assessment of apoptosis by Mitocapture™ assay

MitoCapture™ is a fluorescence-based tool for distinguishing between viable and apoptotic cells by detecting changes in the mitochondrial transmembrane potential. Neutrophils were isolated as above and incubated with appropriate reagent at a concentration of $5x10^4$/ml in a flat-bottomed 96-well plate at 37^{o}C, 5%CO_2 for 2h. MitoCapture™ kit used as per manufacturer's instructions. Briefly, media removed and replaced with Mitocapture™ dye:Mitocapture™ buffer 1:1000 followed by incubation as previous for 30min. Mitocapture™ dye/ buffer then removed and replaced with Mitocapture™ buffer alone. Fluorescence microscopy was performed using a Zeiss Axiovert S100 microscope. Cells fluorescing green were adjudged to have lost mitochondrial membrane potential whilst those fluorescing orange/red were deemed to have intact mitochondria. Three separate experiments with three replicates of individual treatments were performed. Photographs were made and 500 cells were counted per replicate.

2.4.9 Assessment of apoptosis by DNA laddering

DNA was extracted from $10x10^6$neutrophils per treatment. Treatments were performed as before in 2ml eppendorf tubes on a shaking heat block and purified with the use of the DNAeasy kit (Qiagen) according to manufacturer's instructions for isolation of DNA from blood. Briefly: cells centrifuged (5min, 300g), pellet resuspended in 200µl 1xPBS (no cations), 200µl Buffer AL prior to vortexing, and incubation at 56°C for 10min on heat block. Ethanol (200µl, 96–100%) added before further vortexing to obtain a homogeneous solution. Sample loaded to DNeasy Mini spin column placed in a 2 ml

collection tube and centrifuged (6000g, 1min). Flow-through and collection tube discarded before sequential wash-steps with 500µl Buffer AW1 and 500µl Buffer AW2. DNeasy Mini spin column placed in a clean 2ml microcentrifuge tube and DNA eluted with 100µl Buffer AE (spin column centrifuged for 1min at 6000g). DNA concentration and purity was checked with the use of a Nanodrop 100. Approx. 0.5-1µg DNA obtained per treatment. DNA was resuspended in 4x sample buffer and subjected to 1% agarose gel electophoresis as described previously.

2.4.10 Assessment of apoptosis by Apoalert™ assay

A fluorometric caspase activation detection kit (ApoAlert™ kit) was used to measure the kinetics of caspase activation in neutrophils. This kit utilizes caspase substrates attached to fluorescent linker molecules that emit following successful caspase cleavage of substrate. This was carried out in accordance with manufacturer's (Clontech) instructions. Briefly: Neutrophils were incubated as described previously with/without R-roscovitine for times indicated in figure. Cells were pelleted and lysed in 1x cell lysis buffer (manufacturer's own) for 10min on ice. Cells centrifuged at 21000g for 5 min at 4^{o}C and 50µl of cell lysate supernatants was added to a 96-well plate preincubated (37^{o}C, 5min) with 50µl 2xreaction buffer/DTT mix. Plate was incubated for 2h at 37^{o}C and analysed in a fluorescent plate reader (excitation: 380nm, emission: 460nm).

2.4.11 Assessment of apoptosis related proteins by proteome profiler

This was carried out in accordance with manufacturer's instructions. Briefly: each array blocked with Array Buffer 1 in 4-Well Multi-dish (manufacturer's own) for 1h on a rocking platform shaker. Cell lysates as prepared previously added to 1.25 mL of Array Buffer 1 and adjust to a final volume of 1.5ml with lysis Buffer 15. Diluted lysates incubated with array overnight at 4° C on a rocking platform shaker. Array washed with 1X wash buffer and rinsed in distilled water. Followed by 3 further washes with 1X Wash Buffer (10min, rocking platform shaker). Array incubated with detection antibody

cocktail and incubated for 1h followed by further washing steps x3. Array incubated with Streptavidin-HRP for 30min and subsequently washed as previous. Each array exposed to ECL followed by X-ray film requisite time. Apoptosis array data on developed X-ray film quantitated by scanning the film on a transmission-mode scanner and analysis using photoshop image analysis software (Adobe).

2.4.12 Cloning of tat-dn-CDK 7 and 9

DNA was mini-Prepped as per DNAeasy kit (Qiagen) protocol (and see above). DNA was cut with the appropriate restriction enzyme and buffer. The reaction was incubated at the appropriate temperature for the enzyme (usually 37ºC) for a minimum of 4h. Double enzyme restriction digest for directional cloning was carried out in a similar manner, but using an enzyme buffer that gave optimal cutting for both enzymes. DNA could then be isolated by gel purification. Band released by cutting (NcoI/BamHI) was isolated and subjected to PCR reaction to add EcoRI/NotI restriction sites to CDK 7/9 fragment (and ensure in frame)- High fidelity reaction: (94^{o}C (15s), 55^{o}C (30s), 68^{o}C (1min) x30cycles. Reaction recipe: DNA 5µl, Primers FOR and REV (see materials) 2µl, Taq enzyme 1µl, dNTPs.

The PCR product was purified by agarose gel electrophoresis (crystal violet as per topo-xl protocol) and sequencing of PCR product (5µl DNA, 2µl primer) was carried out to ensure fidelity. TOPO XL cloning of the PCR product was as per protocol (Invitrogen). The excised PCR band (CDK7/9 plus restriction enzyme sites) was used in a topoisomerase ligase reaction carried out with 3µl of PCR product to 1µl of tat vector

Plasmid transformed into one-shot bugs, selected by kanamycin (KAN)/ampicillin (AMP) resistance. Colonies were picked and Mini-Prepped. Sequencing was carried out to ensure insertion of CDKdn into TOPO vector. TOPO vector was cut with EcoRI/NotI to release fragment (digestion reaction constituents see above) which was gel purified. The gel purified fragment was cloned into the tat plasmid: tat plasmid cut with

EcorI/NotI, SAP (Shrimp Alkaline Phosphatase) treated and ethanol precipitated. Fragment annealed by DNA ligase reaction (Buffer 1, 2 and 3 (23µl reaction: 7µl insert, 1µl vector, buffers, ddH2O). 5min on ice (Rapid DNA ligation kit Roche).

'One-shot' E.coli transformed with 3 µl pTAT-CDK-DN-His and plated on KAN agar. Colonies picked and mini-prepped (Qiagen protocol). The plasmid was then sequenced bi-directionally with 4 different primers. Once the sequence was confirmed plasmids were maxi prepped and stored at 4^{o}C.

2.4.13 Transformation of *E.coli* BL21

pTat-CDK7/9-DN was transformed into pLys BL21 *E.coli* cells: 3µl of plasmid was added to one aliquot of DH5α cells which spent 30min on ice prior to heat shock at 42^{o}C (45-50s), a further 2min on ice before 200 µl SOCS medium was added. Cells were incubated on orbital shaker (37 ^{o}C) for 1h and cultured overnight on agar/ampicillin (100µg/l) plates at 37^{o}C. Resistant clones (6 on first occasion) were picked (using pipette tip) and grown in L-broth overnight at 37 ^{o}C (50ml tubes, 10ml L-broth). The cells were pelleted at low speed in a centrifuge (2000g) before being resuspended in 100µl of L-broth and spread on to LB-agar plates with the appropriate antibiotic for positive selection. Plates were placed in a 37ºC incubator overnight. DNA was isolated from BL21s and sequenced.

2.4.14 Protein production

Chemically competent BL21 (pLysS DE3) cells were transformed with pTAT-CDK-DN-HIS and selected on antibiotic plates. Colonies were picked and incubated till log phase growth OD600 = 0.6. IPTG induction (1mM). Optimal incubation time and temperature were assessed in small-scale experiments. After 4h incubation at 37^{o}C cells were centrifuged at 50000g and sonicated in 1ml resuspension buffer (see buffers)

before disruption in a cell disruptor. Coomassie gels following centrifugation at 50000g demonstrated protein in pellet fraction. Pellet was washed in isolation buffer (see buffers) prior to resuspension in solubilisation buffer (see buffers). Solubilisation was performed in the presence of protease inhibitor cocktail at 4^{o}C with a magnetic stirrer for 2h. After a further centrifugation step at 50000g supernatant was filtered through a 0.22μM filter prior to batch or HPLC isolation.

2.4.15 Protein isolation (batch method)

As per Clontech Talon system instructions. Briefly, solubilised protein incubated with pre-equilibrated talon resin for 1h at RT or overnight at 4^{o}C. Resin centrifuged at 300g and supernatant retained for analysis. Resin washed with solubilisation buffer x3 and wash supernatant retained for analysis before elution with elution buffer. Samples checked by coomassie staining and western blotting for protein of interest.

2.4.16 Protein purification by immobilised metal affinity chromatography (IMAC) (Refolding on column method)

Filtered protein eluate was loaded onto a 1ml/5ml nickel/cobalt IMAC FF column which had been pre-equilibrated in solubilisation buffer on an ÅKTA liquid chromatography unit. Flow rate was 0.5ml/min and column was washed with 10 column volumes (CV) of solubilisation buffer followed by a 50CV gradient of solubilisation to refolding buffer at 1ml/min. Elution was with 5CV of elution buffer. Fractions (50 ml for run through and wash, 1ml for gradient and elution) were collected for the entire run (Solubilisation buffers 1-3 and respective elution buffers were trialled).

2.4.17 Protein purification by gel filtration and ion exchange

Method 1: Buffer exchange by size exclusion (solubilization buffer components are retarded and the protein will therefore be transferred to a denaturant-free buffer).

A HiPrep 16/60 S200 HR column was used on an ÅKTA liquid chromatography unit. Column was equilibrated in 180ml of refolding buffer and eluted batch sample (containing the solubilized inclusion bodies/proteins in solubilization buffer) was loaded into a 5ml loop (pre-washed with denaturing buffer) and run at 0.2 ml/min with 100% solubilisation buffer, loading 5ml of sample. Fractions (1ml) were collected for the entire run

Method 2: Introducing into the column a decreasing gradient from solubilization buffer to the refolding buffer before sample application. The refolding buffer is then used to elute the protein. This approach should involve a more gradual transfer of the protein to a denaturant-free buffer (Gu et al. 2001).

A HiPrep 16/60 S200 HR column was used on an ÅKTA liquid chromatography unit. Column was equilibrated in 180ml of refolding buffer. Then a gradient was manually run from 0 - 100% solubilisation buffer over 30ml onto the top of the column. Eluted batch sample was loaded into a 5 ml loop (pre washed with solubilisation buffer) and run at 0.2ml/min with 100% solubilisation buffer, loading 5ml of sample. Fractions (1ml) were collected for the entire run

Method 3: Cation exchange
Based on the theoretical isoelectric point of CDK9 we decided to attempt further purification of tat-cdk9dn by cation exchange. Eluted protein was initially desalted by running sample through a HiPrep 26/10 Desalt column prior to cation exchange in HEPES buffer with a 1M NaCl gradient on a MonoS 5/50 GL column.

2.4.18 Coomassie staining of protein gels

Samples were run on 10% gels which were subsequently stained with coomassie dye (see materials) for 30min and destained using destain (see materials) to allow

visualisation of bands.

2.4.19 Glycerol stocks

For long term storage, 8% glycerol stocks of bacterial clones were made by addition of 20µl of a mini prep culture to 1 ml of 8% glycerol in L broth and stored in a minus 80ºC freezer.

2.4.20 Labelling of protein with FITC

A solution of 1mg/ml protein in 0.1M sodium carbonate buffer (pH9) was prepared. To protein solution 50ml of 1mg/ml FITC in DMSO was added in 5ml aliquots whilst continually stirring. Reaction was incubated in dark at 4^{o}C overnight. NH4Cl was added to final concentration of 50mM and incubatded for 2h at 4^{o}C. Xylene cylanol and glycerol were added to 0.1% and 5% respectively. Labelled protein was separated from free FITC by gel filtration through a PD-10 column.

2.4.21 Illumina gene chip assessment of neutrophil transcriptome

This was performed by ARC genomics at Roslin Bioquarter and subsequent bioinformatics data was generated by Dr Jon Manning, CIR Bioinformatics division and the following description of the bioinformatical calculations involved is by him, *"Data output by the proprietary Illumina software were handled using modules of the R-based Bioconductor software suite (www.bioconductor.org). Version 1.14.0 of the 'lumi' package (Du et al. 2008) formed the core of the analysis, providing the routines employed to read, adjust background intensity, transform and normalise the data. The 'variance stabilising transform' suggested by this module's authors was employed in place of the more conventional log-transform, prior to robust spline normalisation. Annotation data were derived initially from an Illumina-provided annotation file, with complementary information taken from the chip-specific 'illuminaHumanv3' and more*

generic 'lumiHumanAll' Bioconductor annotation packages. The level and significance of differential expression for each probe was assessed by use of linear models and empirical Bayes from the 'Limma' package (Smyth 2004) and the resulting p values were adjusted for multiple testing by use of the Benjamini-Hochberg method (Hochberg et al. 1990;Hochberg et al. 1995). Fold changes were derived with the aid the the 'gtools' package. Processed data were stored in a relational database with a dynamic front-end for further analysis".

2.4.22 Administration of bleomycin sulphate and CDK inhibitor drugs *(i.t.)*

Female C57BL/6J mice weighing a minimum of 20g were used for all experiments. The mice were briefly anesthetised using Isofluorane and bleomycin sulphate (Apollo Scientific) administered to the lungs via the trachea, in a volume of 50µl. Following induction of anaesthesia, mice were suspended upright on a purpose-built rig, by resting their teeth on a rigid wire. Their tongues were protruded, and 50 µl of 0.033mg bleomycin sulphate (Apollo Scientific) placed on the oropharynx. The nares of the mice were briefly covered to induce involuntarily aspiration of solution, before mice were returned to the cage to recover from anaesthesia (typically 30s). On day 2 and day 5 post-bleomycin administration, mice (6 per group) were treated I.T. with either 50µl of 0.05% DMSO vehicle control or 10 mg/kg of R-roscovitine or DRB.

2.4.23 Bronchoalveolar lavage (BAL) and histology

Mice were killed 7 days after bleomycin or saline administration with a single 200µl intraperitoneal injection of sodium pentobarbitone (200 mg/ml).The diaphragm was opened via the peritoneal cavity and the rib cage removed. The lungs were cannulated via the trachea *in situ* using a fine cannula which was tied in place with a single suture and BAL was performed using three sequential washes consisting of 0.8 ml ice-cold sterile 0.9% saline. All samples were centrifuged at 180g for 5min at 4°C, the supernatants removed and the cell pellets resuspended in 0.5ml sterile 0.9% saline. Total

cell numbers were counted using a nucleocounter and cytocentrifuge smears prepared and stained with Diff Quik™ for differential cell-counts to be made. At least 300 cells per slide were counted and the results expressed as total number of neutrophils and macrophages in the lung lavage. Histological analysis of the 7 day experiments was undertaken without BAL to maintain tissue integrity and lung injury was assessed by histological examination of paraffin-embedded lung sections stained with hematoxylin and eosin.

2.4.24 Statistical analyses (excluding illumina data, see above)

All experiments were performed at least three times unless stated otherwise and each treatment done in triplicate and the results are expressed as the mean ± SEM. Data were analysed by a one-way ANOVA with a Student Newman-Keuls multiple comparison *post hoc* test with a 95% confidence interval (multiple conditions) or paired t-test (2 conditions) as appropriate (InStat software).

3. Results: CDK inhibitor drugs drive neutrophil apoptosis

The CDK inhibitor drug R–roscovitine was shown to drive apoptosis of human neutrophils by Rossi et al in their 2006 Nature Medicine paper (Rossi et al. 2006). This finding was interesting because, as discussed in the introduction, neutrophils are terminally differentiated cells and ought not to have functional cell-cycle machinery. It might be expected that cell-cycle inhibiting drugs would have no effect on neutrophils or alternatively promote survival as is the case in terminally differentiated neuronal cells (Maas, Jr. et al. 1998). The precise mechanisms by which CDK inhibitor drug driven neutrophil apoptosis is promoted have not previously been elucidated. The following chapter comprises an investigation of CDK inhibitor drug driven neutrophil apoptosis in order to offer mechanistic insight into the inflammation resolving properties of these pharmacological agents. It begins with a careful delineation of the time-course and pharmacokinetics of CDK inhibitor drug driven neutrophil apoptosis as determined by morphological assessment of cytocentrifuge preparations and flow cytometric assessment of annexin-V/PI binding and uptake. These same techniques are used to compare the effects of CDK inhibitor drugs with known modulators of neutrophil apoptosis. Subsequently caspase involvement (western blotting and fluorometric assay) and mitochondrial assessment are performed in attempt to dissect the apoptotic pathway involved in the process. Confocal microscopy and live cell imaging as well as DNA ladder detection are also employed to provide a comprehensive assessment of apoptosis. These experiments provide important evidence for the mechanism of action of CDK inhibitor drugs in resolution of inflammation by showing why neutrophils are specifically driven to apoptosis by these pharmacological agents.

3.1 R-roscovitine promotes neutrophil apoptosis in a time-dependent manner

It is important to thoroughly investigate the chronology of apoptosis induction by pharmacological agents for a number of reasons. Firstly, it is necessary to establish that true apoptosis is induced in the absence of necrosis and that any necrosis detected is secondary to apoptosis. Careful time-course experiments assessing both apoptosis and necrosis should establish this convincingly. Secondly, some agents (e.g., TNF-α (Cross et al. 2008;Murray et al. 1997)) can have dual effects on neutrophil longevity by promoting apoptosis in an early phase but subsequently prolonging neutrophil lifespan or vice-versa (e.g., MG-132 (Derouet et al. 2006;Ward et al. 1999)). Thirdly, there is significant variation in the reported rates of constitutive apoptosis in the published literature (Sabroe et al. 2004) and therefore carefully controlled experiments are of paramount importance.

In figures 3.1 and 3.2, flow cytometry assessment of annexin-V/PI staining and morphological assessment of Diff-Quik stained cytocentrifuge preparations were performed to document the concentration-dependency and time-course of CDK inhibitor drug driven neutrophil apoptosis. Annexin-V binds to PS externalised as one of the hallmarks of apoptosis and postulated to play a role in allowing subsequent apoptotic cell engulfment by phagocytes. PI binds DNA and is used as a marker of loss of plasma membrane integrity and therefore necrosis.

Timeline experiments (Figure 3.2) demonstrate that R-roscovitine promotes a significant increase compared to control in the proportion of neutrophils annexin-V positive/PI negative by 4-8h (Figure 3.2 iii and iv). The majority of neutrophils treated with R-roscovitine are annexin-V positive/PI negative by 8h (75.0±2.7%) and by 20h annexin-V positive/PI positive cells are the largest population proportionally (60.5±2.1%). Time-course experiments are shown to demonstrate that cells transition through annexin-V

positivity to dual annexin-V/ PI positivity. This indicates that R-roscovitine primarily drives apoptosis and that the emergence of a necrotic cell population at late time-points (as in figure 3.1) is a secondary phenomenon. Time-lines showing constitutive fate of neutrophils (Figure 3.2 i and ii), in our laboratory using our methodology, are shown for comparison given the considerable heterogeneity of such data in the literature (Sabroe et al. 2004). In figure 3.2b representative flow plots and cytocentrifuge preparation images are shown.

Figure 3.1a: Representative flow plots and cytocentrifuge preparations showing time-dependent effect of R-roscovitine on neutrophil apoptosis

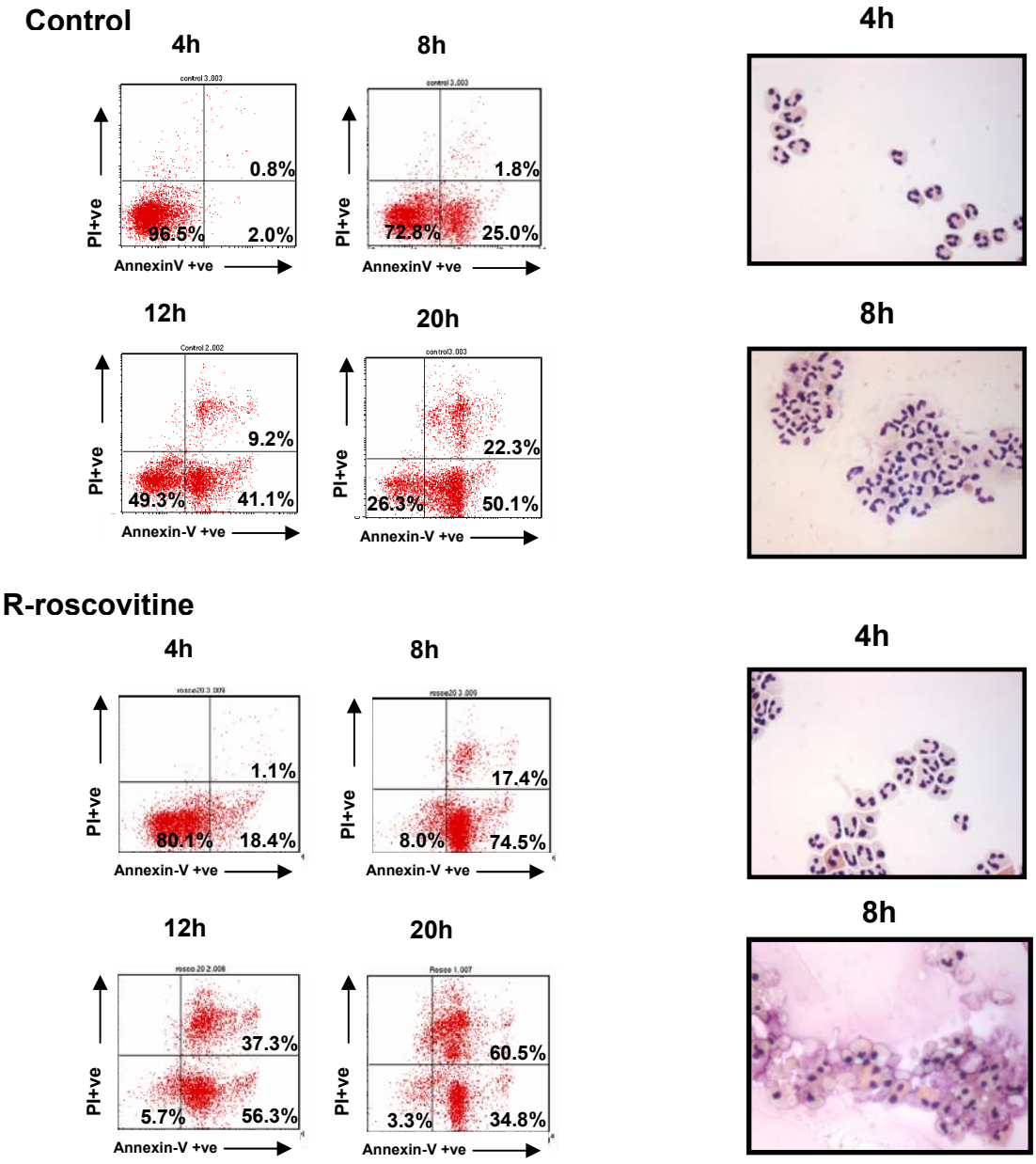


*Figure 3.1a**:** Time courses for neutrophil viability and death in the presence of R-roscovitine (20μM) as assessed by flow cytometric measurement of annexin-V (FL-1)/PI (FL-2) positivity are shown as representative flow cytometry plots from each time-point. Flow plots are interpreted as follows: lower left quadrant annexin-V-ve/PI –ve (viable), lower right quadrant annexin-V+ve/PI-ve (apoptotic), upper right quadrant annexin-V+ve/PI+ve (necrotic) (n=6). Photomicrographs (x400) of neutrophil morphology at 4 and 8h for neutrophils cultured alone and with R-roscovitine (20μM) treatment. Viable neutrophils have multi-lobed nuclei while apoptotic cells have round, densely packed 'pyknotic' nuclei. At 8h with R-roscovitine some 'ghost cells' with no nuclei are visible.*

Figure 3.1b: Time-course showing effect of R-roscovitine on neutrophil viability over 20h

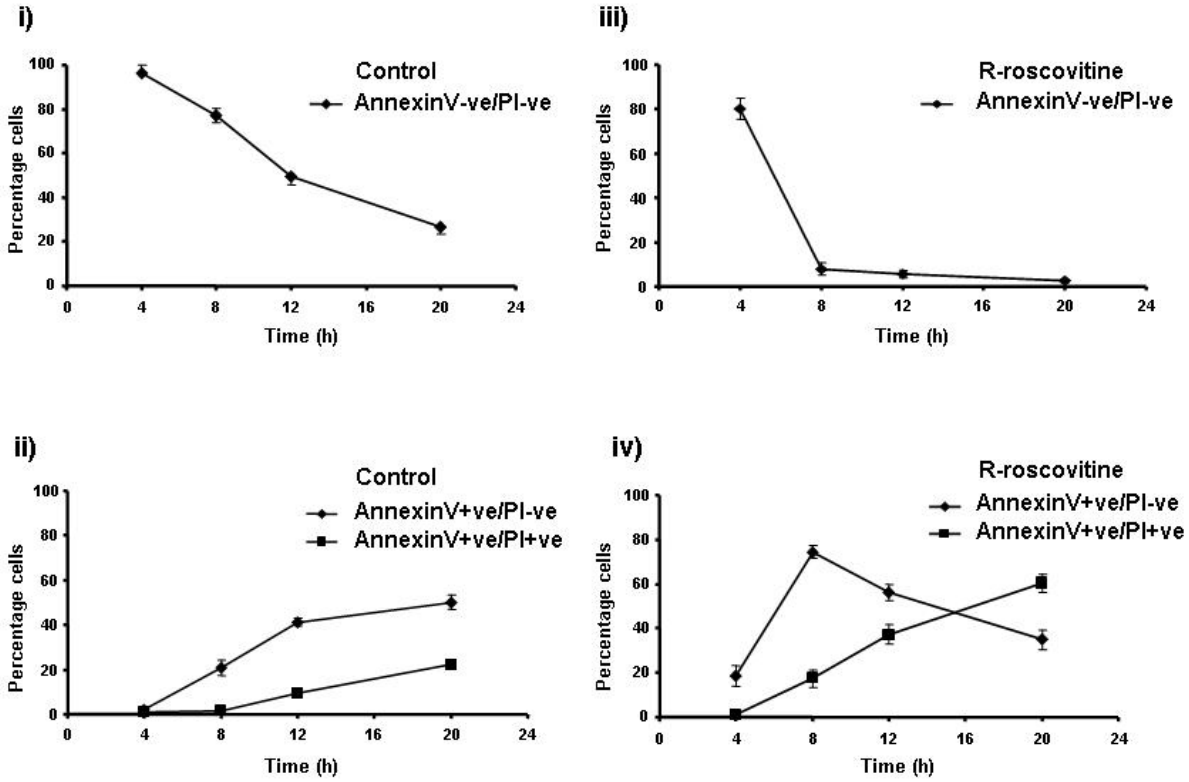


Figure 3.1b: Neutrophils were cultured at 5x10^6/ml in IMDM containing 10% autologous serum and R-roscovitine at a concentration of 20μM. At 4, 8, 12 and 20h samples of cultured neutrophils were removed for generation of cytocentrifuge preparations for examination of morphological changes associated with apoptosis and for preparation of samples for flow cytometric analysis of Annexin V binding and PI staining. Data are presented as a time-course showing the change in untreated neutrophil viability (defined as Annexin V-ve/PI -ve) in (i), apoptosis (Annexin V+ve/PI-ve) and necrosis (Annexin V+ve/PI+ve) both depicted in (ii) over a 20h time period. The effect of R-roscovitine on the various populations as defined above is shown in (iii) and (iv). Lines showing development of apoptosis and necrosis are superimposed to demonstrate that necrosis follows apoptosis (secondary necrosis).

Figure 3.2: Concentration-dependent effect of the CDK inhibitor, R-roscovitine, on neutrophil viability

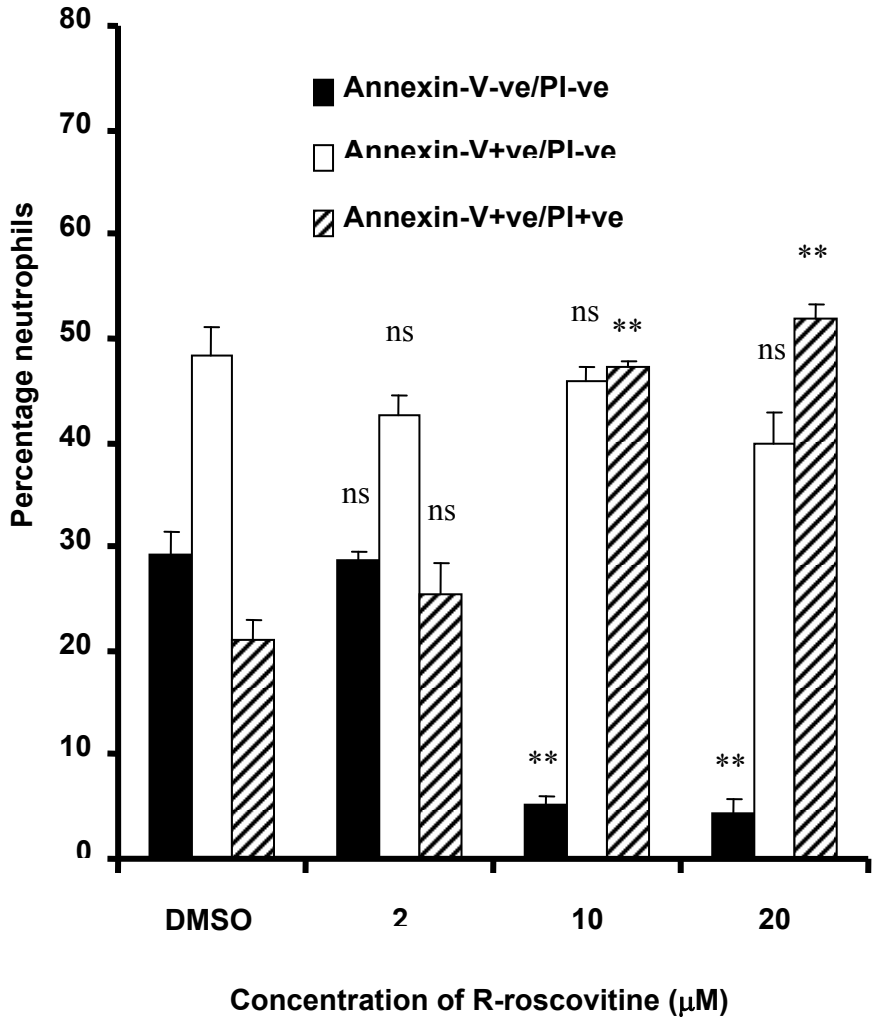


*Figure 3.2: Neutrophils were cultured at 5x10^6/ml in IMDM containing 10% autologous serum and either DMSO (vehicle control) or R-roscovitine at concentrations of 2, 10 or 20μM. At 20h cultured neutrophils were removed for generation of cytocentrifuge preparations for examination of morphological changes associated with apoptosis and for preparation of samples for flow cytometric analysis of Annexin-V binding and PI staining. Data showing the change in untreated neutrophil viability (defined as Annexin-V-ve/PI-ve), apoptosis (Annexin-V+ve/PI-ve) and necrosis (Annexin-V+ve/PI+ve) with increasing concentration of R-roscovitine. Statistical comparisons are to vehicle control by ANOVA with post-hoc Student Newman-Keuls, p<0.01**.Data shown are mean +SEM from n= 3 experiments.*

R-roscovitine at 10-20μM concentration does significantly decrease the proportion of annexin-V negative/PI negative cells compared to control. R-roscovitine 2μM had no statistically significant effect on any cell population. At a concentration of 10μM or 20μM, R-roscovitine did not appear to increase the proportion of annexin-V positive/PI negative cells compared to control at 20h (Figure 3.2) but the proportion of annexin-V positive/PI positive cells was significantly increased. This suggests either that a population of cells are dying primarily by necrosis or that more cells become annexin-V positive at an earlier time-point and subsequently become PI positive. The interpretation of these findings is either that the dual positive cells are 'late apoptotic' (secondary necrosis) or that they are primarily necrotic. Interestingly, R-roscovitine appears to have a steep effective concentration range with almost no effects at 2μM and only slightly enhanced efficacy (dual positive cells slightly increased, not statistically significant) at 20μM as compared to 10μM.

In order to provide additional evidence and support for the reliability of our flow cytometry and cytocentrifuge preparation data, live confocal microscopy imaging of neutrophils treated with R-roscovitine and progressively incorporating annexin-V and subsequently PI was performed. Stills are shown in figure 3.3 and the video is available in appendix 1. In this video neutrophil viability is monitored over 8h by annexin-V/PI fluorescence. Annexin-V incorporation occurs 314min post R-roscovitine treatment and loss of plasma membrane integrity denoted by PI staining of the nucleus happens at 415min. This gives a window of approx. 1h 40min to allow phagocyte clearance of the apoptotic neutrophil. Live imaging by light microscopy was also performed and is available in appendix 2.

Figure 3.3: Live cell imaging of neutrophil treated with R-roscovitine

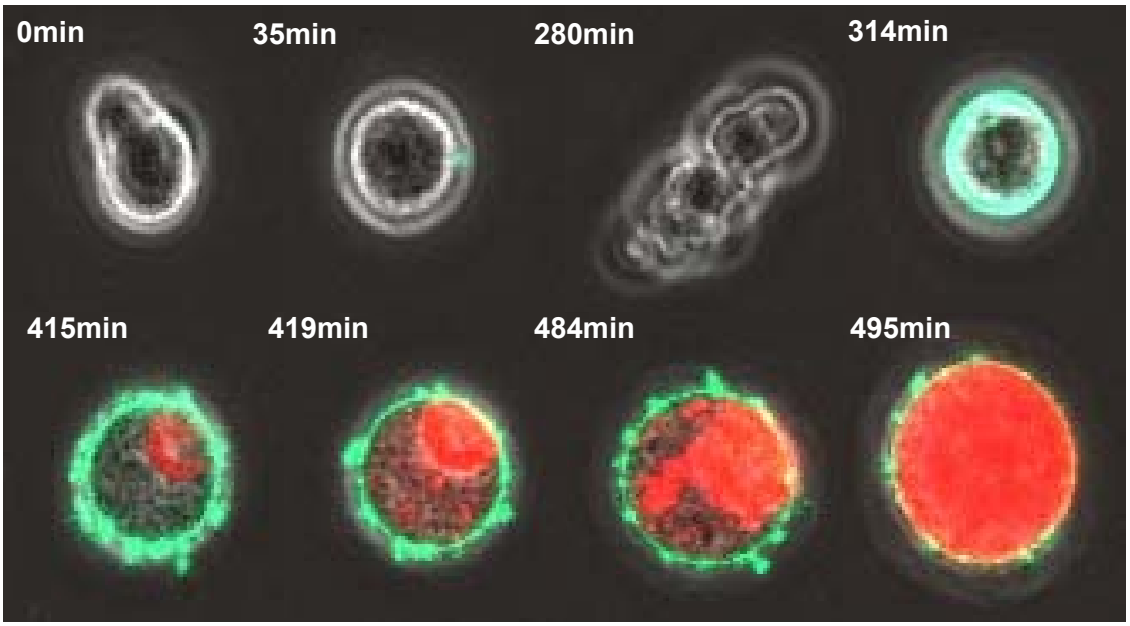


Figure 3.3: Live cell imaging by confocal microcopy of neutrophils undergoing apoptosis and secondary necrosis in response to R-roscovitine (20μM). Representative stills are shown corresponding to significant events in the death process. At 0min the neutrophil is still mobile and alive. At 35min the neutrophil rounds up and becomes stationary. At 280min the neutrophil begins the 'blebbing' process. At 314min the outer membrane of the neutrophil becomes annexin-V positive demonstrated by green peripheral fluorescence. Between 415min and 495min the neutrophil undergoes the process of secondary necrosis demonstrated by the red fluorescence of PI in the nucleus. The nuclear membrane expands or becomes permeable at 484min. Images made with a Zeiss LSM510 confocal microscope with the use of a POCmini live cell imaging chamber. Magnification x63 Images were captured at 2 minute intervals with 488nm and 543nm lasers.

3.2 A comparison of CDK inhibitor drugs and other described modulators of apoptosis

A number of pharmacological agents have been demonstrated to modulate neutrophil apoptosis and it seemed reasonable therefore to compare the effects of CDK inhibitor drugs with these well-described compounds. Both pro-apoptotic and anti-apoptotic agents were investigated in order to highlight the extent to which neutrophil fate can be influenced by external interventions. A number of compounds were tested including dexamethasone (Meagher et al. 1996), cycloheximide (Whyte et al. 1997), db-cAMP (Martin et al. 2001), gliotoxin (Ward et al. 1999) actinomycin-D (Whyte et al. 1997) (figures 1.4 and 1.5) and a panel of CDK inhibitors (data shown for CDK4 inhibitor and DRB), figures 3.6 and 3.7). CDK4 inhibitor had no pro-apoptotic effect within its effective concentration range suggesting that this kinase may not be a relevant target in terms of neutrophil apoptosis. The effect of R-roascovitine (CDK2, 5, 7, 9 specific) and DRB (CDK7, 9 specific) was similar to that observed with the protein synthesis inhibitors actinomycin-D and cycloheximide (Whyte et al. 1997) so a time-course experiment was performed to further clarify the pharmacological kinetics (figure 3.5).

In order to examine the impact of CDK inhibitor drugs on neutrophil survival in the presence of survival factors known to be abundant at sites of inflammation neutrophils were treated with LPS and TNF-α in combination with R-roscovitine (figure 3.8). At 20h the survival effects of these two agents that signal through the key neutrophil survival signaling pathway, the NF-κB pathway (Ward et al. 1999), were overcome by CDK inhibitor drugs. As TNF-α is known to have an early pro-apoptotic effect that is mediated through activation of the extrinsic ‘death receptor’ pathway of apoptosis (Murray et al. 1997) I was keen to investigate the effect of CDK inhibitor drugs on this property. Interestingly, R-roscovitine enhanced the early pro-apoptotic effects of TNF-α in a similar fashion to gliotoxin though co-treatment with both agents promoted more significant levels of apoptosis (figure 3.9).

Figure 3.4: Comparison of the effects of various agents on neutrophil viability at 20h

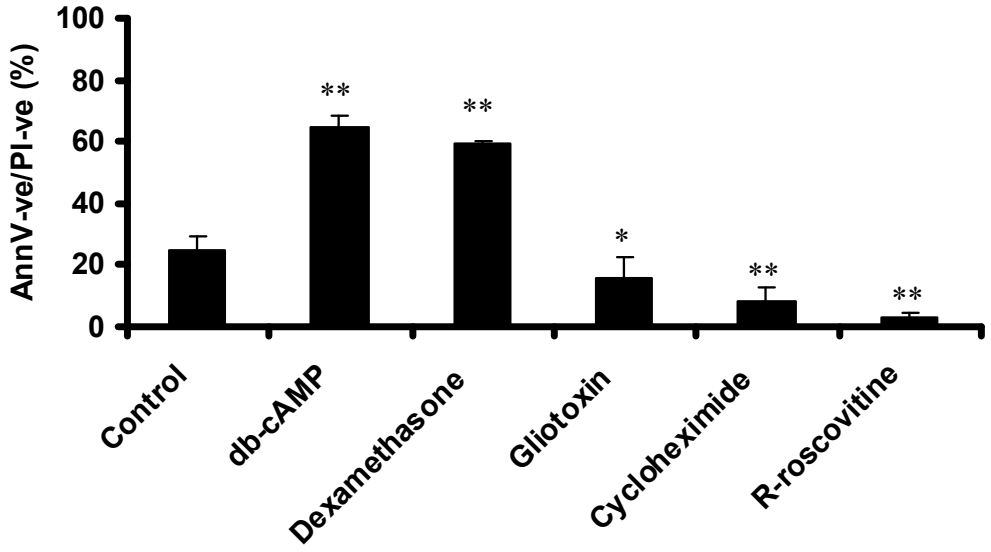


Figure 3.4: Neutrophils were cultured at $5x10^6$/ml in IMDM containing 10% autologous serum and R-roscovitine (20μM), dexamethasone (0.1μm), db-cAMP (0.2mM), gliotoxin (0.1μg/ml) or cycloheximide (10μM). At 20h samples of cultured neutrophils were removed for generation of cytocentrifuge preparations for examination of morphological changes associated with apoptosis and for flow cytometric analysis of annexin-V binding and PI staining. Data are presented as a bar chart showing the change in untreated neutrophil viability (defined as annexin V-ve/PI -ve). Chart is representative of n=3 experiments of 3 separate replicates and are presented as mean+/-SEM. Statistical significant difference from control indicated by p<0.05= and p<0.01=** by ANOVA with Student Newman-Keuls multiple comparison post hoc test with a 95% confidence interval.*

Figure 3.5: Time-courses of neutrophil viability following treatment with actinomycin-D or R-roscovitine

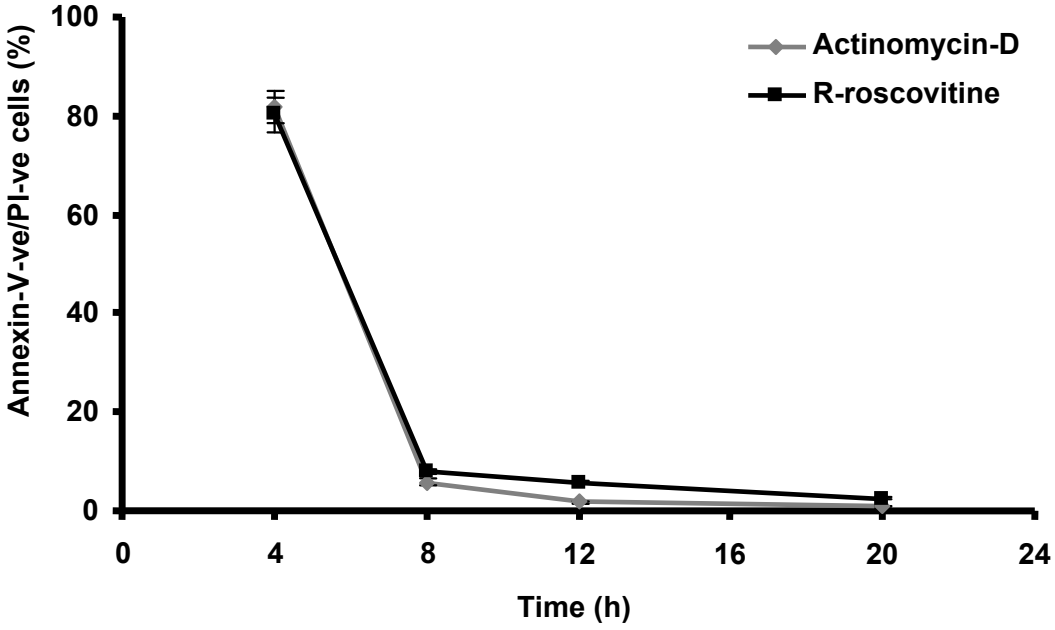


Figure 3.5: Neutrophils were cultured at 5x10^6/ml in IMDM containing 10% autologous serum and R-roscovitine (20μM) or actinomycin-D (10μM). At 4, 8, 12 and 20h samples of cultured neutrophils were removed for generation of cytocentrifuge preparations for examination of morphological changes associated with apoptosis and for flow cytometric analysis of annexin-V binding and PI staining. Data are presented as a time-course showing the change in untreated neutrophil viability (defined as annexin-V-ve/PI-ve) over a 20h time period. Data are representative of n=3 experiments of 3 separate replicates and are presented as mean+/-SEM.

Figure 3.6: Comparison of the effects of DRB and R-roscovitine on neutrophil viability over a 20h time-course

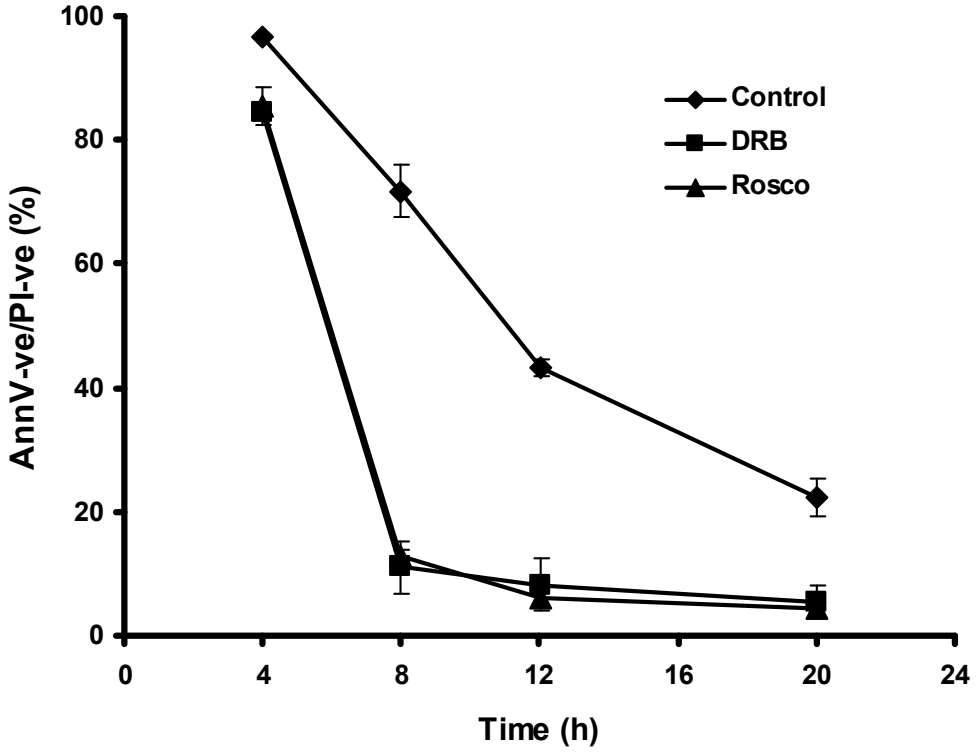


Figure 3.6: Neutrophils were cultured at 5x10^6/ml in IMDM containing 10% autologous serum and R-roscovitine (20μM) or DRB (20μM). At 4, 8, 12 and 20h samples of cultured neutrophils were removed for generation of cytocentrifuge preparations for examination of morphological changes associated with apoptosis and for flow cytometric analysis of annexin-V binding and PI staining. Data are presented as a time-course showing the change in untreated neutrophil viability (defined as annexin-V-ve/PI -ve) over a 20h time period. Data are representative of n=3 experiments of 3 separate replicates and are presented as mean+/-SEM.

Figure 3.7: Effects of CDK4 inhibitor on neutrophil viability at 12 and 20h time points

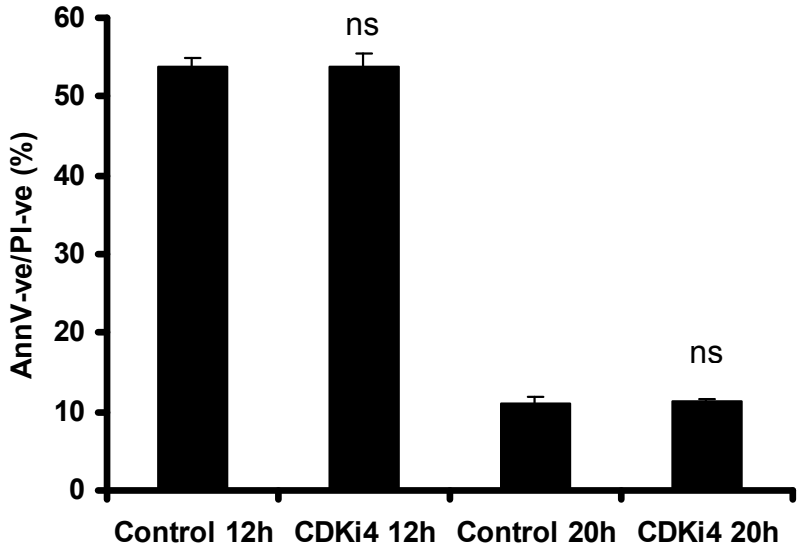


Figure 3.7: Neutrophils were cultured at 5x10^6/ml in IMDM containing 10% autologous serum and CDK4 inhibitor (CDKi4,100nM). At 12 and 20h samples of cultured neutrophils were removed for generation of cytocentrifuge preparations for examination of morphological changes associated with apoptosis and for flow cytometric analysis of annexin-V binding and PI staining. Data are presented as a bar chart showing the change in neutrophil viability (defined as annexin V-ve/PI –ve of neutrophils treated with CDK4 inhibitor compared to control (untreated). Data are representative of n=3 experiments of 3 separate replicates and are presented as mean+/-SEM. No significant difference between CDK 4 inhibitor and control (paired t-test).

Figure 3.8: CDK inhibitor drugs overcome the survival effects of LPS and TNF-α on neutrophils

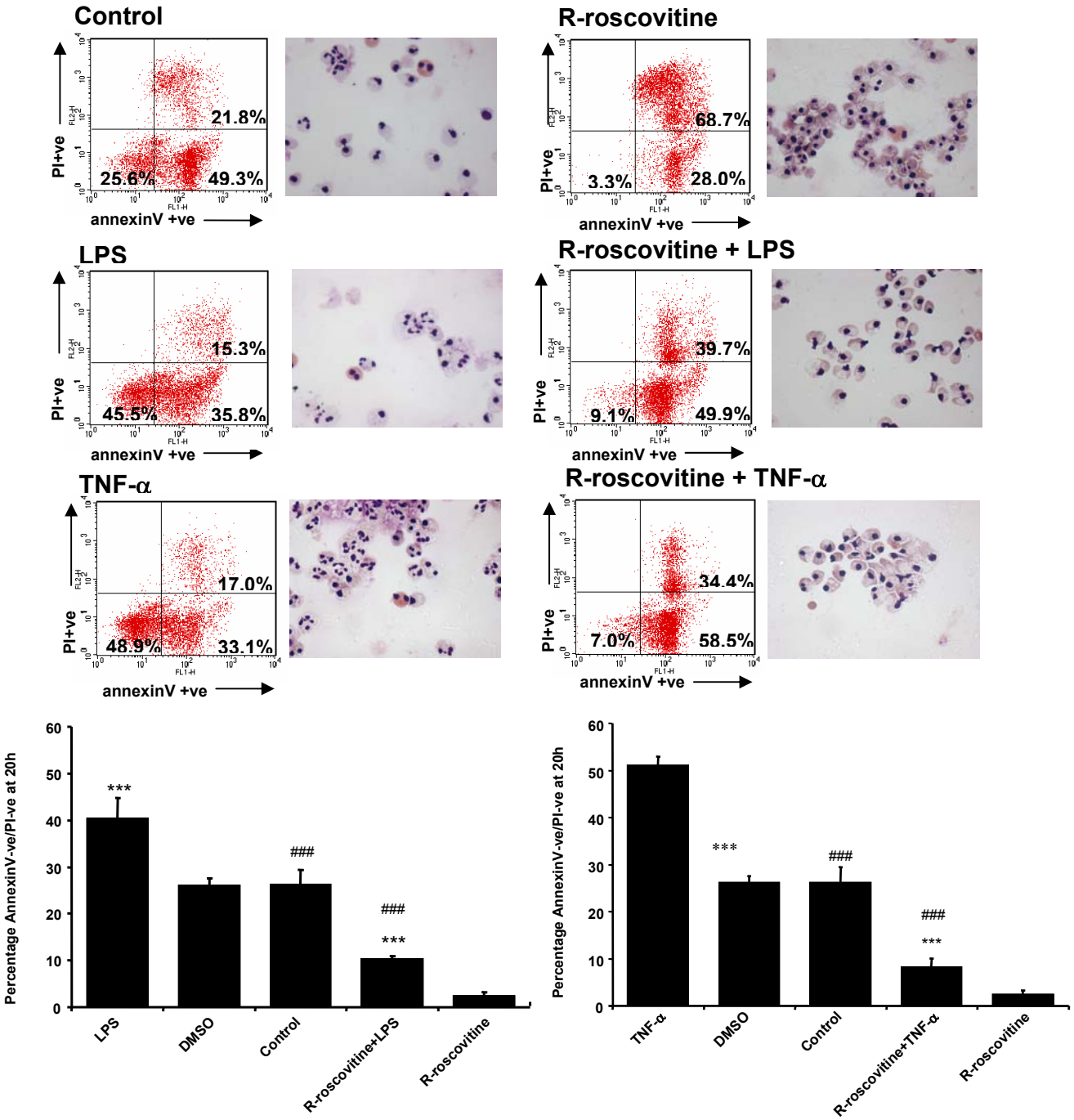


*Figure 3.8: Neutrophils were cultured at 5x10^6/ml in IMDM containing 10% autologous serum alone as control (i) or with R-roscovitine (20μM) (ii), LPS (100ng/ml) (iii), R-roscovitine + LPS (iv), TNF-α (10ng/ml) (v), or R-roscovitine + TNF-α (vi) as indicated above for 20h. Apoptosis was assessed using annexin-V/ PI staining and confirmed by morphological examination under light microscopy. Results represent n=6, with each experiment being performed in triplicate. Representative flow cytometry plots (lower left quadrant is annexin-V-ve/PI-ve, lower right is annexin-V+ve/PI-ve and upper right is annexin-V+ve/PI+ve) are shown along with examples of neutrophil morphology from the appropriate experimental condition. Plots show effect of R-roscovitine on neutrophil viability in the presence of LPS (vii) and TNF-α (viii). Statistically significant difference to p<0.001 between LPS/TNF-α and R-roscovitine + LPS/ R-roscovitine + TNF-α is shown as ***. Statistically significant difference to p<0.001 between R-roscovitine + LPS/ R-roscovitine + TNF-α and control is shown as ### (ANOVA with a Student Newman-Keuls multiple comparison post hoc test with a 95% confidence interval).*

Figure 3.9: R-roscovitine enhances the early pro-apoptotic effects of TNF-α

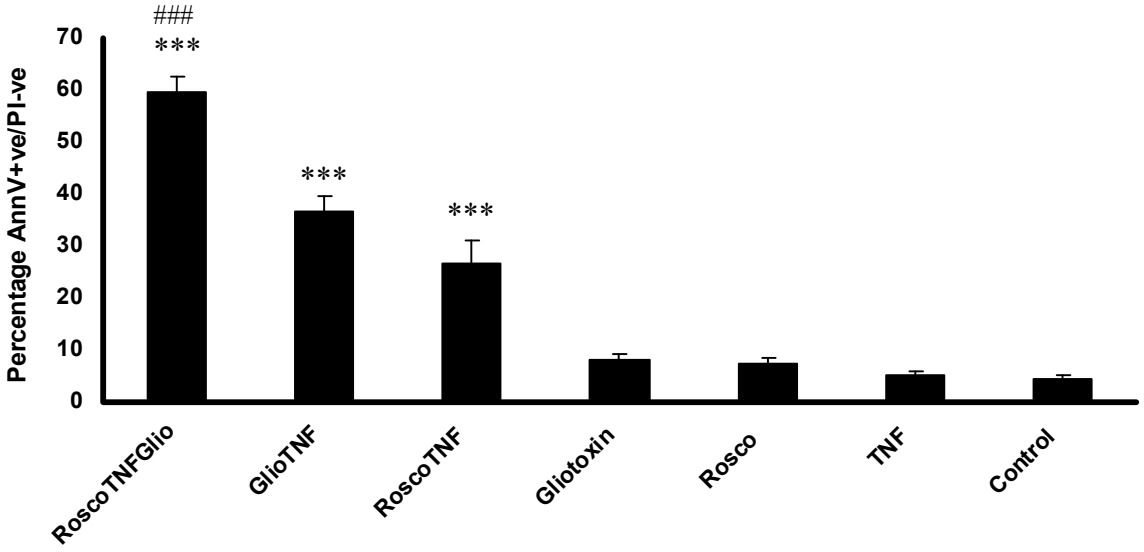


*Figure 3.9: Neutrophils were cultured at $5x10^6$/ml in IMDM containing 10% autologous serum alone as control or with R-roscovitine (Rosco, 20μM), Gliotoxin (Glio, 0.1μg/ml), TNF-α (TNF, 10ng/ml), or combinations of these agents as indicated above for 2h. Apoptosis was assessed using annexin-V/ PI staining and confirmed by morphological examination under light microscopy. Results represent n=3, with each experiment being performed in triplicate. Statistically significant difference to control $p<0.001$ = *** and between RoscoTNFGlio and GlioTNF/RoscoTNF = ### (ANOVA with a Student Newman-Keuls multiple comparison post hoc test with a 95% confidence interval).*

3.3 CDK inhibitor drugs promote activation of caspases

Caspases are pivotal enzymes of the apoptotic process and are generally activated by two separate, though linked, pathways (Hallett et al. 2008b). Caspase-1 has also been implicated in pyroptosis a postulated novel form of cell death related to microbial infection (Kepp et al. 2010).

In brief (see figure 1 and description in text of introduction for more detail), caspase-8 can be recruited and activated by death receptor ligand binding (classically TNF-α or fas-ligand (FasL) resulting in progression through the 'extrinsic pathway' of apoptosis. Alternatively, cellular stressors (such as genotoxic drugs or UV) can lead to an imbalance of pro- vs anti-apoptotic bcl-2 homologues leading to excess pro-apoptotic homologues and perturbation of mitochondrial membrane integrity. Loss of mitochondrial membrane integrity leads to apoptosome formation and activation of caspase-9 in the 'intrinsic pathway' of apoptosis. In type II apoptotic cells such as neutrophils these pathways are linked by caspase-8 activation of Bid leading to mitochondrial membrane integrity loss and signalling via both apoptotic pathways. In both pathways the terminal executioner caspase is caspase-3. In order to characterize the predominant apoptotic pathway that occurs in response to CDK inhibitor drugs it was important to consider this property.

In figure 3.10 I show by western blotting that caspases -3, -8 and -9 are cleaved (activated) in response to CDK inhibitor drugs though it proved difficult to separate these events chronologically. Figure 3.11, representative images of confocal microscopy for caspase-3 cleavage in neutrophils treated with R-roscovitine is supportive of my western blotting data. Additionally in figure 3.12 I have used a fluorometric caspase activation detection kit (ApoAlert^{TM} kit) in the hope that this might be a more sensitive measure of the kinetics of caspase activation in neutrophils. This kit utilizes caspase substrates attached to fluorescent linker molecules that emit following successful caspase cleavage of substrate. The strongest and most reliable signal was for the terminal executioner caspase, caspase-3 which may suggest that initiator caspases need

Figure 3.10: CDK inhibitor drugs activate caspases to promote apoptosis

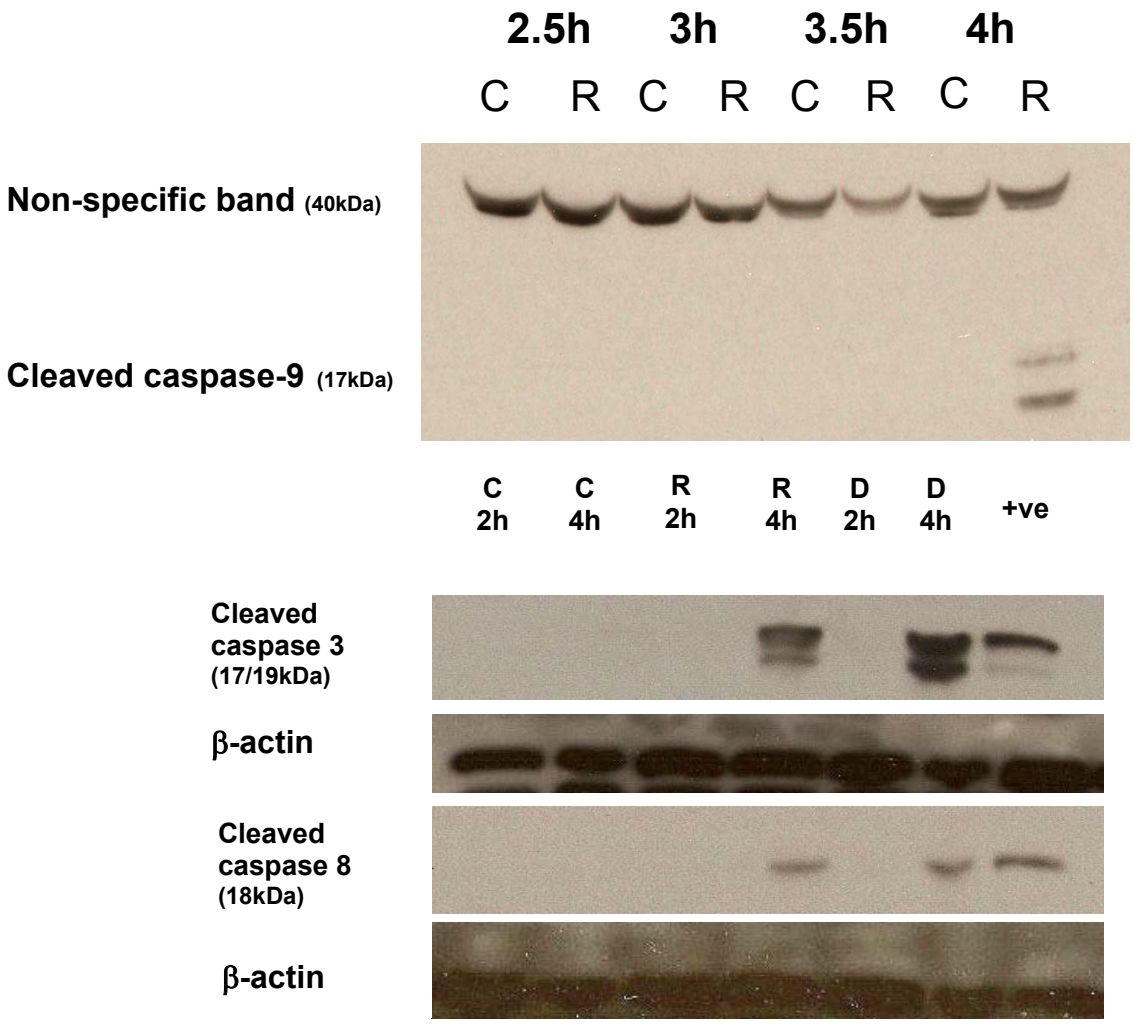


Figure 3.10: Western blotting for cleaved (active) caspases 3,8 and 9. Neutrophils were incubated with appropriate agents (media alone (C), R-roscovitine 20μM (R), DRB 20μM (D)) in 2ml eppendorfs on a shaking heat block at 37°C for 2-4h and subsequently lysed using standard neutrophil lysis buffer. Positive control (+ve) was neutrophils left for 24h at 37°C. Blots above are representative of n=3 experiments.

Figure 3.11: Confocal microscopy of caspase-3 cleavage in neutrophils incubated with R-roscovitine

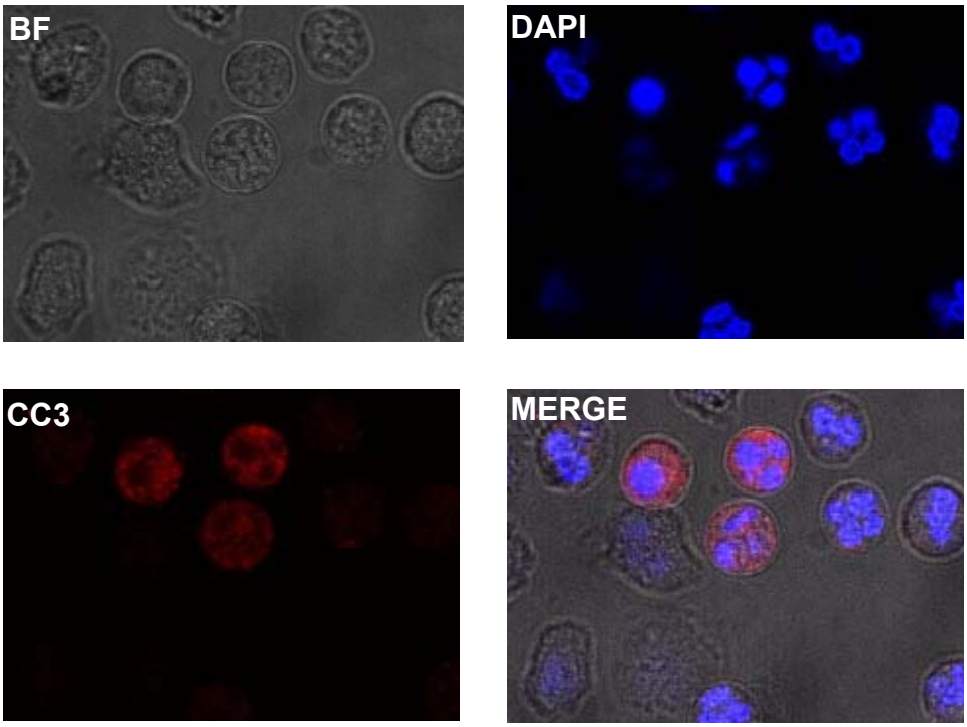


Figure 3.11: Confocal microscopy of neutrophils labelled for cleaved caspase-3 by indirect immunofluorescence. Neutrophils were incubated at $5x10^6$/ml for 4h with R-roscovitine (20μM). Upper left panel shows transmitted light/bright field image (BF). Upper right panel shows DAPI staining of nuclear material. Lower left panel shows staining for cleaved caspase-3 (CC3). Lower right panel is a merged image (MERGE). Leica SP microscope (x630 oil immersion, UV and 633nM HeNe lasers). Representative image.

Figure 3.12: Multiple caspases activated by CDK inhibitor drugs

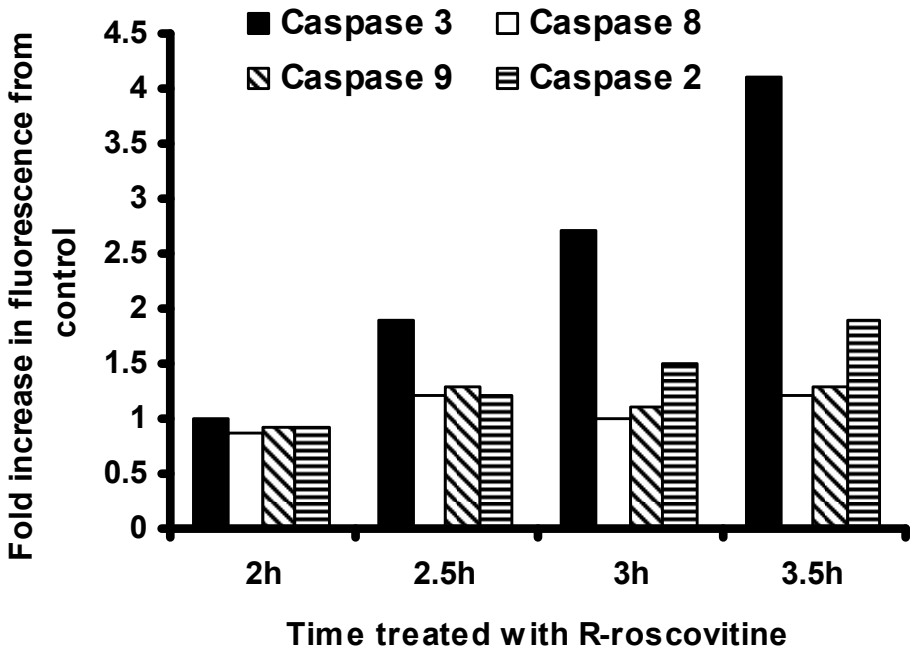


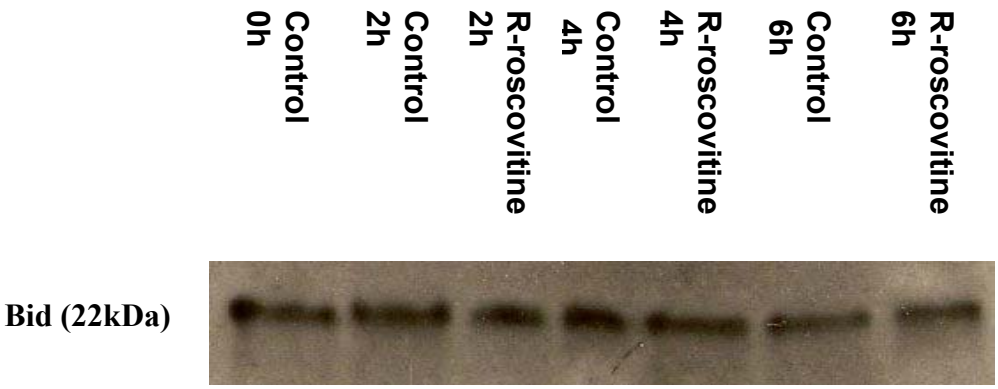
Figure 3.12: ApoAlert kit- Caspase cleavage in neutrophils in response to treatment with R-roscovitine. Neutrophils were prepared as previously described and lysed at times indicated in accordance with manufacturers (Clontech)) instructions. Fluorescence was measured on a fluorescent plate reader (excitation: 380nm, emission: 460nm). Results represent n=2 experiments (No statistics performed).

only be activated to a comparatively lesser degree than the executioner. This seems compatible with a model that uses caspases-8 and -9 as triggers whilst caspase-3 is functionally important in the successful conclusion of the apoptotic process.

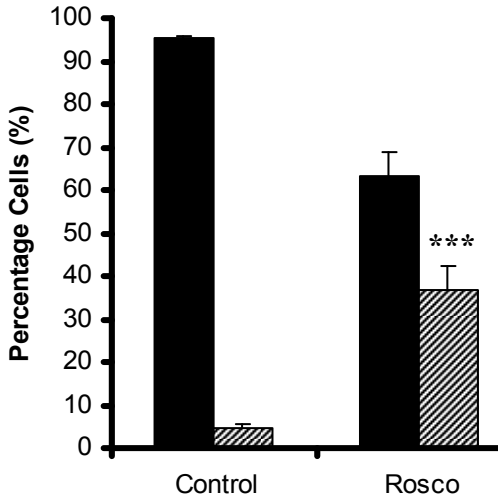
In order to further assess the relevant apoptotic pathways we investigated mitochondrial membrane integrity and also Bid cleavage (figure 3.13). If it can be shown that mitochondrial outer membrane permeabilisation is present prior to the appearance of the morphological features of apoptosis and that Bid cleavage is not detectable it seems reasonable to assume (in a type II apoptotic cell) that the critical apoptotic pathway is the intrinsic pathway. Mitochondrial permeability was assessed with the use of Mitocapture^{TM} dye. The dye accumulates in mitochondria of viable cells and fluoresces orange/red whilst in apoptotic cells it leeches out of permeabilised mitochondria into the cytoplasm where it fluoresces green. We were able to demonstrate significant induction of mitochondrial permeabilisation by R-roscovitine at early time-points (2.5h) where there is no evidence of morphological changes of apoptosis. Western blotting for the pro-apoptotic protein Bid (figure 3.13) revealed no evidence of cleavage throughout the time-range where apoptosis was shown to occur in earlier figures.

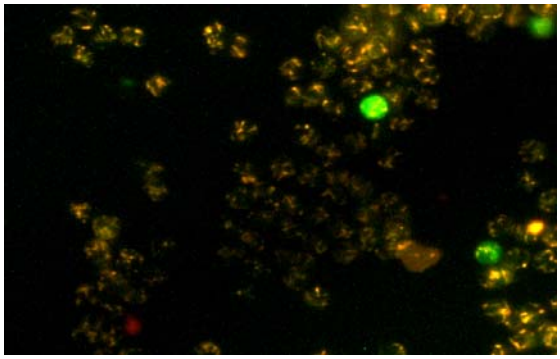
Figure 3.13: CDK inhibitor drugs promote intrinsic pathway apoptosis

i)



ii)





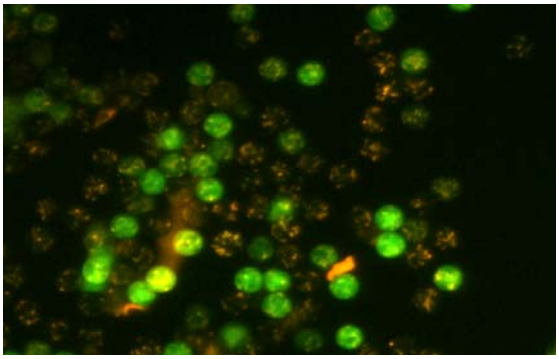


*Figure 3.13: In i) neutrophils were incubated with medium alone or plus R-roscovitine (20μM) and subjected to standard neutrophil lysis at time-points between 0-6h prior to immunoblotting for full-length Bid. In ii) neutrophils were treated with medium alone or plus R-roscovitine (20μM) for 2h then incubated with Mitocapture™ according to manufacturer's instructions (30min) and examined by fluorescent microscopy (x320). The dye fluoresces red/orange in the mitochondria of viable neutrophils (Solid bars) and green in the cytoplasm of neutrophils with loss of mitochondrial transmembrane potential (Striped bars). All experiments (n=3). Statistically significant difference in the percentage of cells that have lost mitochondrial membrane integrity between R-roscovitine and control to $p<0.001$ shown as *** by paired t-test.*

3.4 CDK inhibitor drug treatment of neutrophil granulocytes leads to classical internucleosomal DNA laddering

A classical measure of apoptosis which is under-represented in recent literature is the demonstration of internucleosomal cleavage of DNA at intervals of 200bp resulting in the classical 'DNA ladder' effect (Wyllie 1980). The process of apoptosis causes DNA cleavage in a typical internucleosomal fragmentation pattern by the enzyme Caspase activated DNase (CAD) which is normally inhibited by the inhibitor of CAD (ICAD)/DNA fragmentation factor (DFF)-45 complex (Widlak et al. 2005). The presence of DNA laddering is determined by DNA isolation and gel electrophoresis. Figure 3.14 demonstrates a typical DNA ladder from human neutrophils treated with R-roscovitine for 6h.

Figure 3.14: R-roscovitine induces classical DNA laddering in association with the induction of apoptosis in human neutrophils

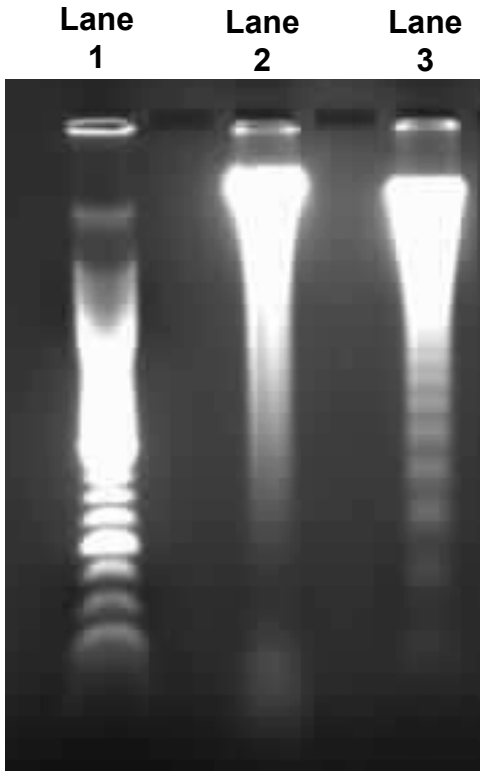


Figure 3.14: Neutrophil DNA (approx. 300ng in 4x sample buffer per lane) isolated as per methods () run on 1% agarose gel in TBE for 1.5h. Lane 1 is 100bp marker, Lane 2 is control untreated neutrophils at 6h and Lane 3 is neutrophils treated with R-roscovitine (20μM) for6h incubation. Image captured with UV light camera and 'Grabit' software.

3.5 Discussion

In this chapter the mechanisms by which apoptosis proceeds in human neutrophils have been investigated. This work adds to the literature characterizing the process of apoptosis in these cells and significantly adds to our knowledge of the effects of CDK inhibitor drugs on neutrophil apoptosis.

It has been established that CDK inhibitor drugs primarily induce apoptosis in neutrophils by careful delineation of the time-line of neutrophil death. This is important because it removes any doubt that a population of cells stimulated to undergo apoptosis by CDK inihibitor drugs (identified by PS expression visualized by annexin-V binding), subsequently undergo secondary necrosis (identified by loss of plasma membrane integrity and visualized by PI incorporation to nucleus). Of interest, live-cell imaging demonstrates that neutrophils remain apoptotic for 1-2h before undergoing secondary necrosis which is illustrative of the demands placed on the mononuclear phagocyte system's clearance capacity by the resolution of significant neutrophilic inflammation. It is clear that the time-course for this event may be significantly altered *in vivo* depending on the environmental conditions that the cell is exposed to. However, it is not necessarily the case that the time an apoptotic cell can persist without becoming necrotic would be shorter *in vivo*. In this confocal microscopy experiment the cell is periodically exposed to lasers to allow visualization of fluorescence (although time-course seems comparable to other *in vitro* data) and the population of cells is probably comparatively sparse. There are a number of environmental conditions that may significantly impact on neutrophil longevity and that may differ between *in vitro* and *in vivo* work including: hypoxia (Hannah et al. 1995;Leuenroth et al. 2000a), cell density (Hannah et al. 1998), temperature (Pryde et al. 2000), cell-cell contact and adhesion (Coxon et al. 1996;Ginis et al. 1997)

To reinforce flow cytometry and cytocentrifuge preparation morphological data with regard to CDK inhibitor drug induced neutrophil apoptosis a further 6 techniques

(western blotting for caspases (figure 3.10), Apoalert™ assay (figure 3.12), indirect immunofluorescence (figure 3.11), live-cell imaging (figure 3.3), mitochondrial outer membrane permeabilisation assay (figure 3.13) and DNA laddering (figure 3.14)) are employed. It has been recommended by an international committee that the demonstration of apoptosis should entail multiple experimental approaches and careful use of nomenclature in order to achieve reliable end-points (Galluzzi et al. 2009;Kroemer et al. 2009). This is because a wide variation in the assessment of apoptosis exists in the literature and some features of the apoptotic process are common to other modes of cell death. In addition the absence of some key features of apoptosis such as caspase cleavage may not necessarily denote cell viability (Kroemer et al. 2005) while PS externalisation has been shown to be reversible albeit under very specific experimental conditions (Geske et al. 2001). This chapter addresses those concerns with the series of experiments it documents. These experiments support apoptosis as the primary method of neutrophil death in response to CDK inhibitor drugs and give insight into the mechanisms by which apoptosis progresses.

CDK inhibitor drugs disturb mitochondrial membrane potential and activate caspases. Although caspase activity has been demonstrated it proved difficult to reliably separate the individual elements of the caspase cascade, a failing that is presumably due to the rapid procession of this event to conclusion. However, it is clear that an early event in CDK inhibitor driven neutrophil apoptosis is mitochondrial outer membrane permeabilisation and it is also clear that the pro-apoptotic protein Bid is not cleaved over the time-course examined (figure 3.13). As previously discussed, neutrophils are Type II apoptotic cells and therefore can link the extrinsic to the intrinsic apoptotic pathway by Bid cleavage. Truncated bid which is activated by caspase-8 cleavage can catalyse the permeabilisation of the outer mitochondrial membrane (Li et al. 1998). These results suggest that this does not occur during CDK inhibitor drug treatment of neutrophils which in turn suggests that apoptosis proceeds via the intrinsic pathway. This finding is consistent with results from the published literature that investigated CDK inhibitor drugs in cancer cell-lines (Lahusen et al. 2003;MacCallum et al. 2005).

Additionally, the inflammatory agent TNF-α which is known to activate the extrinsic pathway of neutrophil apoptosis by signalling through the TNF receptor induces apoptosis within 2h of exposure a time-course that is inconsistent with the data for CDK inhibitor drugs (Murray et al. 1997). Interestingly the combination of TNF-α and R-roscovitine produces enhanced apoptosis at 2h (figure 3.9) a finding that has previously been demonstrated with the NF-κB inhibitor gliotoxin (Ward et al. 1999). This result is interesting because it suggests that activation of the extrinsic pathway of apoptosis is not mediated by R-roscovitine but that inhibition of TNF-α induced production of survival proteins does occur. This finding has important ramifications for the mechanism of action of R-roscovitine that will be discussed in later chapters. At sites of inflammation neutrophils have enhanced longevity because they are exposed to agents such as TNF-α, LPS, fMLP and certain other cytokines (Haslett et al. 1985;Haslett et al. 1991;Lee et al. 1993). It has been a matter of some debate whether this exposure stimulates enhanced transcription of key survival proteins or merely promotes stabilisation of previously synthesised proteins. It was even debated (and still is in some quarters) whether neutrophils had transcriptional capacity at all or whether following differentiation they relied on pre-packaged proteins or perhaps mRNA for their functionality (Cassatella et al. 1995;McDonald et al. 1997;McDonald et al. 1998). The relative contribution of neutrophils and monocytes/macrophages to the inflammatory milieu in terms of cytokine production signalling is still unclear (Cassatella 1995;Cassatella et al. 1997;Cassatella 1999;Nathan 2006;Sabroe et al. 2004). Whilst monocytes/macrophages undoubtedly have greater transcriptional capacity neutrophils often outnumber them so heavily that any capacity for signal generation could conceivably tip the balance. The reality under physiological conditions is likely to be that these cells don't work against each other but in concert and the outcome of an inflammatory scenario is a balance of their inputs.

In an attempt to mirror the *in vivo* exposure of neutrophils to inflammatory agents I have performed experiments that demonstrate CDK inhibitor drugs can over-ride the effects of the survival agents TNF-α and LPS to promote neutrophil apoptosis. This is an

interesting finding as both these agents stimulate the NF-κB signalling system which is undoubtedly a central survival pathway for human neutrophils (McDonald et al. 1997;Ward et al. 1999). Indeed the NF-κB pathway has previously been identified as a key target of CDK inhibitor drugs in cancer cell-lines (Dey et al. 2008). In cancer cell-lines it was shown that R-roscovitine could influence upstream I-kappa-B-kinase (IKK) signalling by an unknown mechanism leading to down-regulation of key genes under the control of the activated NF-κB transcription factor. We have previously investigated the relevance of this pathway to CDK inhibitor drug induced neutrophil apoptosis but could find no conclusive evidence for any significant, direct involvement (Leitch et al. 2010b). Yet it is clear from our data with R-roscovitine and TNF-α that an enhanced apoptotic effect similar to that seen with the NF-κB inhibitor gliotoxin occurs. However, it should be noted that co-incubation of these inhibitors with TNF-α led to an even greater effect on neutrophil apoptosis which to some extent suggests different mechanisms of action.

By comparison of the pharmacokinetic action of R-roscovitine with other agents known to influence neutrophil longevity, I have attempted to gain some insight into possible mechanism of action. It is known that neutrophils subjected to protein synthesis inhibition undergo enhanced apoptosis at early time points (Whyte et al. 1997). This has been used as evidence for the central importance of key survival proteins such as Mcl-1 to neutrophil life-span (Edwards et al. 2004b). R-roscovitine has a similar time-course of action to actinomycin-D and cycloheximide and there is a precedent in the literature for an effect of CDK inhibitor drugs on the transcription of survival proteins (Kapasi et al. 2008;MacCallum et al. 2005;McClue et al. 2002). Indeed, it is known that CDK7 (Roy et al. 1994) and 9 (Zhu et al. 1997) play important roles in the initiation and elongation of selected mRNA transcripts by RNA pol II the key enzyme in human transcriptional machinery. Additionally, MacCallum et al have demonstrated that two survival proteins of known importance to neutrophils, Mcl-1 and XIAP, are influenced by inhibition of CDKs 7 and 9 (MacCallum et al. 2005). It seemed reasonable therefore to further investigate both the effect of CDK inhibitor drugs on Mcl-1 and the presence and

functionality of CDKs in neutrophils. The results of these investigations are presented in the following chapters.

3.6 Summary of Chapter 3

1. **CDK inhibitor drugs induce time-dependent apoptosis in human neutrophils** *as demonstrated by flow cytometry assessment of annexin-V/PI, morphological assessment, western blotting for caspases, DNA laddering, confocal microscopy and assessment of mitochondrial membrane potential.*
2. **The apoptosis induced by CDK inhibitor drugs proceeds via the intrinsic pathway** *as demonstrated by western blotting for tBid and assessment of mitochondrial membrane potential*
3. **Apoptosis induced by CDK inhibitor drugs has similar kinetics to that of the transcriptional inhibitor actinomycin-D** *as assessed by flow cytometry*

4. Results: Mcl-1 down-regulation by CDK inhibitor drugs drives neutrophil apoptosis

The previous chapter shows that CDK inhibitor drugs drive classical neutrophil apoptosis by the intrinsic pathway. This implicates an important protein family termed the 'bcl-2 homologues'. These proteins have considerable structural homology but are split into two groups with directly opposing actions. The pro-apoptotic bcl-2 homologues such as Bad, Bax and Bim are thought to facilitate loss of mitochondrial membrane potential by forming pores in the outer mitochondrial membrane. They are counterbalanced by the anti-apoptotic bcl-2 homologues such as Bcl-2, Mcl-1, A1 and Bcl-xl that are thought to sequester pro-apoptotic homologues away from mitochondria (Brenner et al. 2009). An imbalance in the levels of these opposing groups leads to early apoptosis or extended longevity. There is controversy over which are the most relevant proteins from this family to neutrophil longevity and apoptosis. It has been stated that Bcl-2 is not present in neutrophil granulocytes and there is a large body of work that places Mcl-1 in the central role usually occupied by Bcl-2 (Moulding et al. 2001;Villunger et al. 2003). The most important pro-apoptotic bcl-2 homologues have previously been thought to be Bax and Bid but new work seems to indicate that Bim is of prime importance (Andina et al. 2009;Cowburn et al. 2010). In this chapter I intend to investigate the effects of CDK inhibitor drugs on the bcl-2 homologue family with special emphasis on the anti-apoptotic protein Mcl-1. Mcl-1 is assessed by western blotting in the presence of factors that are known to alter neutrophil longevity or Mcl-1 half-life. The effects of preserving Mcl-1 on neutrophil apoptosis are investigated by flow cytometry for annexin-V binding and PI incorporation. For comparison protein and mRNA levels of other bcl-2 homologue family members are obtained by western blotting, proteome profile array, semi-quantitative PCR and illumina gene chip technology. The NF-κB regulated survival protein XIAP is investigated at the mRNA and protein level.

It is my contention that Mcl-1 is in the vanguard of apoptotic decision-making in neutrophil granulocytes and that CDK inhibitor drugs are capable of profoundly down-regulating this protein to drive neutrophil apoptosis.

4.1 R-roscovitine down-regulates Mcl-1, circumvents GM-CSF-mediated protection of Mcl-1 and, in combination with TNF-α or LPS, enhances Mcl-1 down-regulation.

Edwards and colleagues have previously shown that levels of Mcl-1 protein begin to decline by 4h and that the cytokine GM-CSF has a protective effect against Mcl-1 decline (Derouet et al. 2004). Figure 4.1i) confirms this finding and also demonstrates that R-roscovitine further down-regulates levels of Mcl-1 in neutrophils at 4h. This figure also demonstrates the ability of R-roscovitine to over-ride the protective effect of LPS, TNF-α and GM-CSF on Mcl-1 (figure 4.1i). However, when R-roscovitine was co-incubated with TNF-α levels of Mcl-1 were reduced more substantially than with R-roscovitine alone (Figure 4.1i).

In addition the proteasome inhibitor MG-132 which is known to preserve Mcl-1 in untreated neutrophils could also prevent Mcl-1 degradation in neutrophils treated with CDK inhibitor drugs. This effect was more significant than that conferred by the pan-caspase inhibitor zVAD-fmk (figure 4.1ii).

Figure 4.1: CDK inhibitor drugs down-regulate the survival protein Mcl-1

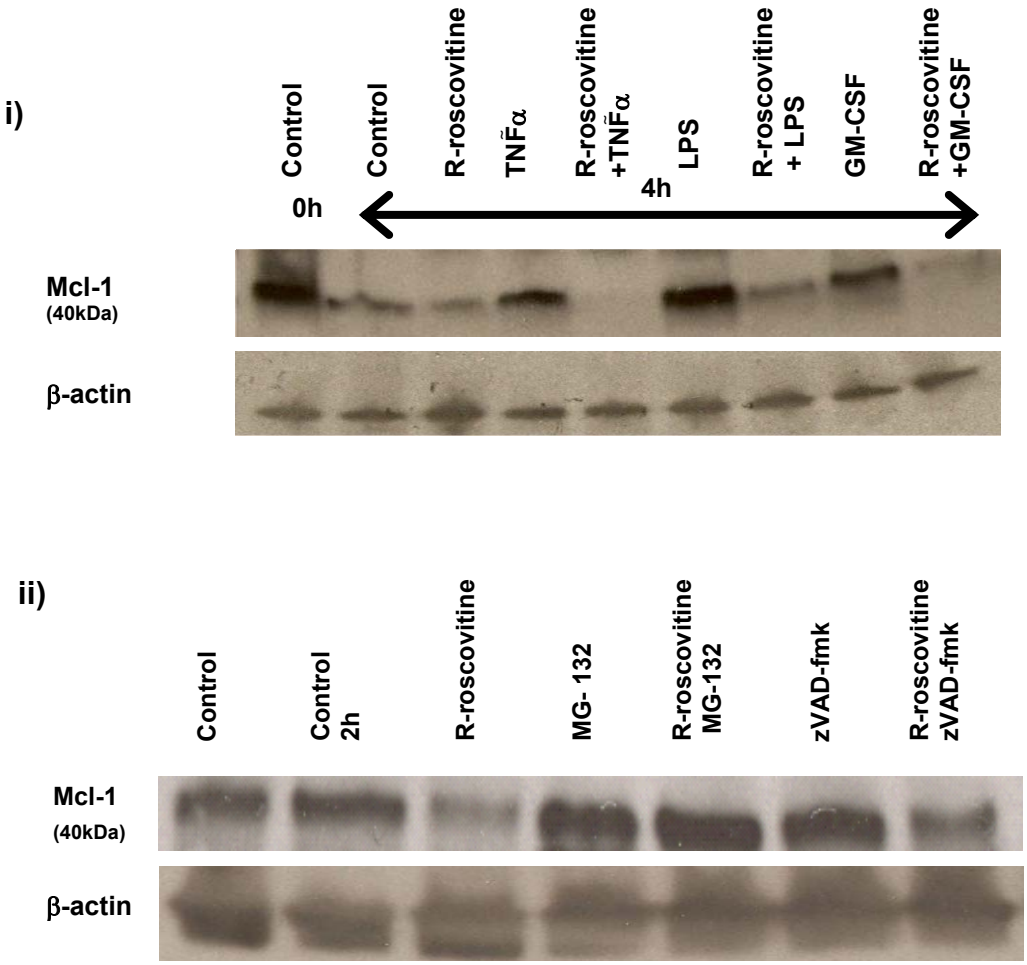


Figure 4.1: In i) Neutrophils at $5x10^6$/ml in IMDM containing 10% autologous serum were either lysed immediately (time 0) or incubated for 4h in either the absence (control) or presence of GM-CSF (50ng/ml), LPS (100ng/ml), TNF-α (10ng/ml) or R-roscovitine (20μM) and immunoblotted for Mcl-1 or β-actin.. In ii), Neutrophils at $5x10^6$/ml in IMDM containing 10% autologous serum were either lysed immediately (time 0) or incubated for 2h in either the absence (control) or presence of MG132 (50μM), zVAD-fmk (100μM), R-roscovitine (20μM) or combinations of these agents then lysed and immunoblotted for Mcl-1 or β-actin. Blots shown are representative of 3 experiments.

4.2 Mcl-1 down-regulation by CDK inhibitor drugs is prevented by proteasome inhibition and short-term neutrophil survival increased

Induction of neutrophil apoptosis by R-roscovitine (20μM) was evident at 4h and by 20h the majority of cells had undergone apoptosis and were noted to be secondarily necrotic. This process was documented by flow cytometric analysis and confirmed by light microscopic morphological examination (Figure 3.1a and b). The effect of R-roscovitine was time-dependent.

I hypothesized that if the mechanism of action of R-roscovitine driven neutrophil apoptosis was related to an effect on transcription it might be prevented or at least delayed by the stabilization of Mcl-1. This might be achieved by transfection/transduction but these are difficult techniques to apply to neutrophils. Co-treatment with a proteasome inhibitor was a simple, if non-specific approach to testing the hypothesis which might be further validated by supportive experimental evidence if it proved successful. Mcl-1 is an unusual bcl-2 homologue in that it has a PEST domain which targets it for degradation in the proteasome in under 2h (Edwards et al. 2004b). Figure 4.2 shows that R-roscovitine-mediated annexin-V incorporation can be reversed up to 12h by co-incubation with the proteasome inhibitor MG-132 or the pan-caspase inhibitor zVAD-fmk. A significant proportion of neutrophils co-treated with zVAD-fmk and R-roscovitine were resistant to annexin-V incorporation even at 20h.

In figure 4.3 it can be seen that prevention of annexin-V incorporation with MG-132 was only evident if the drug was administered within 2h of R-roscovitine treatment, a time-course consistent with a transcription-based mechanism of action. By contrast at 4h and 6h post treatment with R-roscovitine the pan-caspase inhibitor zVAD-fmk is still able to preserve some cells from annexin-V incorporation.

Additionally, figure 4.4 shows that preservation of Mcl-1 by proteasome inhibition in CDK inhibitor treated neutrophils maintains mitochondrial outer membrane integrity which accords with the proposed mechanism of action of Mcl-1 (see introduction). By contrast despite preventing annexin-V incorporation mediated by CDK inhibitior drugs even at late-time-points zVAD-fmk has no effect on the maintenance of mitochondrial integrity.

Figure 4.2: The proteasome inhibitor MG-132 can delay apoptosis induced by the CDK inhibitor drug, R-roscovitine

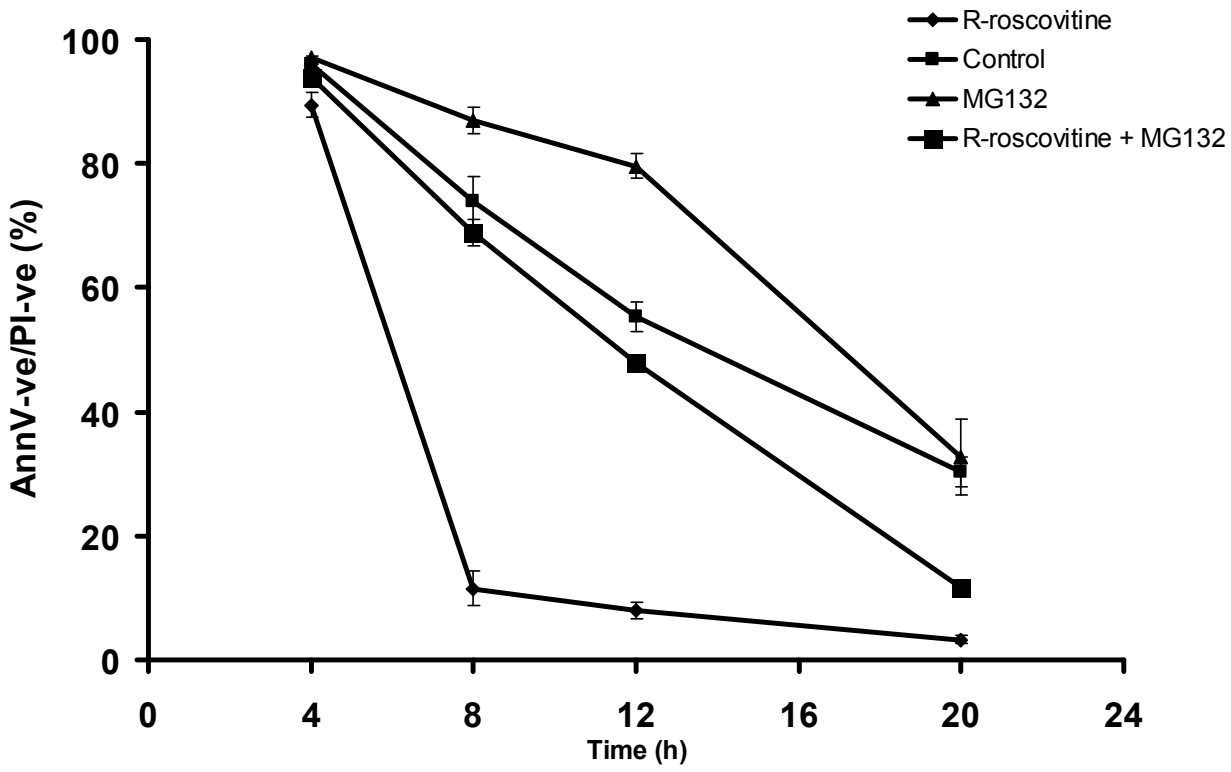


Figure 4.2: Neutrophils were cultured as described previously with no agents (control), R-roscovitine (20μM), MG-132 (50μM) or with a combination of R-roscovitine and MG-132 at the same concentrations. Cell viability was subsequently assessed as previously described using flow cytometry for annexin-V/PI positivity and morphological examination of cytocentrifuge preparations.

Figure 4.3: Delayed proteasome inhibition fails to prevent R-roscovitine-induced neutrophil death at 8h

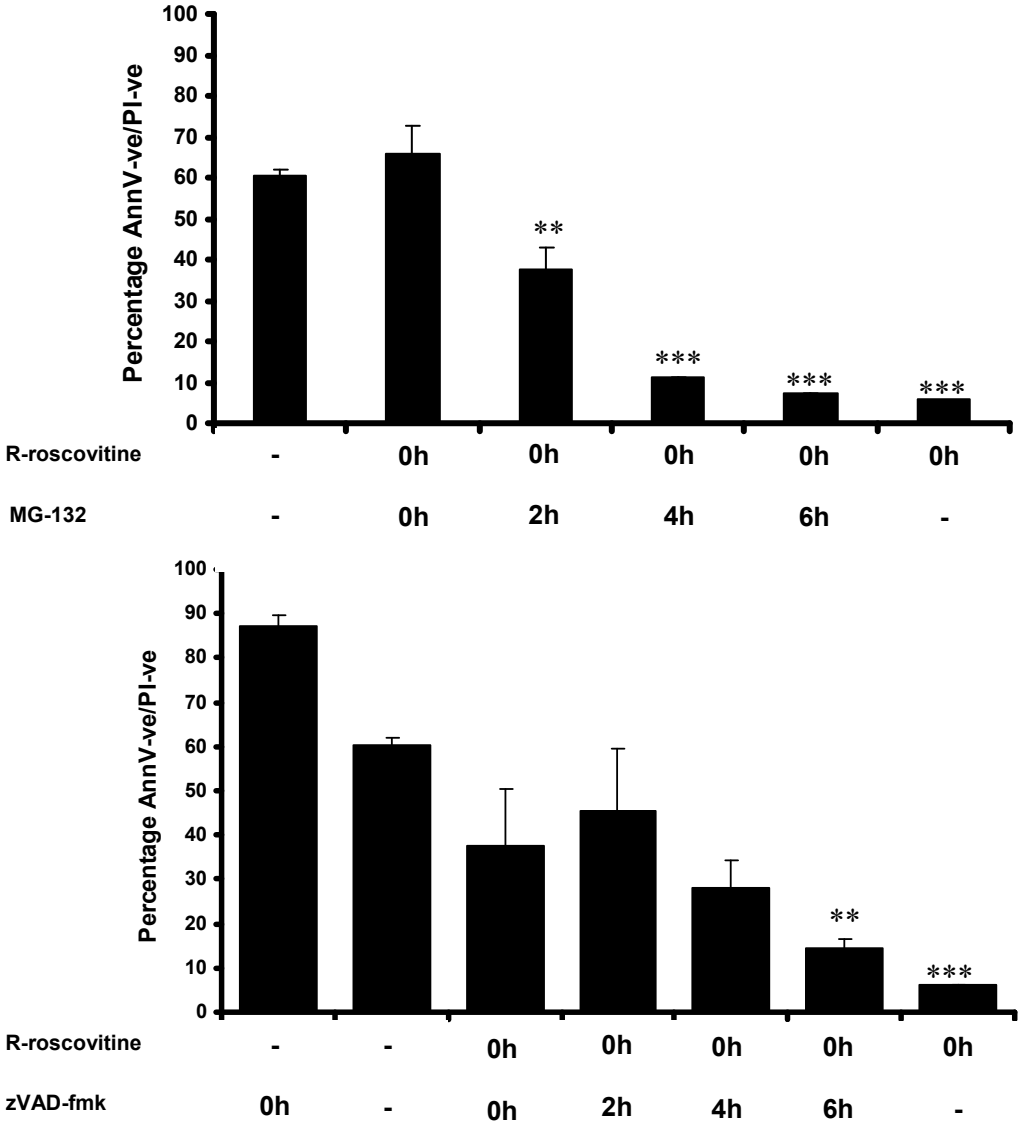


*Figure 4.3: Neutrophils were cultured as described previously for 8h with no agents (control) or R-roscovitine (20μM). MG-132 (50μM) or z-VAD-fmk (100μM) were administered at 0h,2h,4h or 6h in order to assess the time dependency of these agents ability to prevent annexin-V incorporation in neutrophils treated with R-roscovitine. Cell viability was subsequently assessed as previously described using flow cytometry and cytocentrifuge examination. Statistically significant differences are shown as $p<0.01$** and $p<0.001$=*** compared to combination of R-roscovitine and MG132 or R-roscovitine and zVAD-fmk by ANOVA with a Student Newman-Keuls multiple comparison post hoc test with a 95% confidence interval.*

Figure 4.4: Proteasome inhibition prevents changes in mitochondrial transmembrane potential induced by R-roscovitine

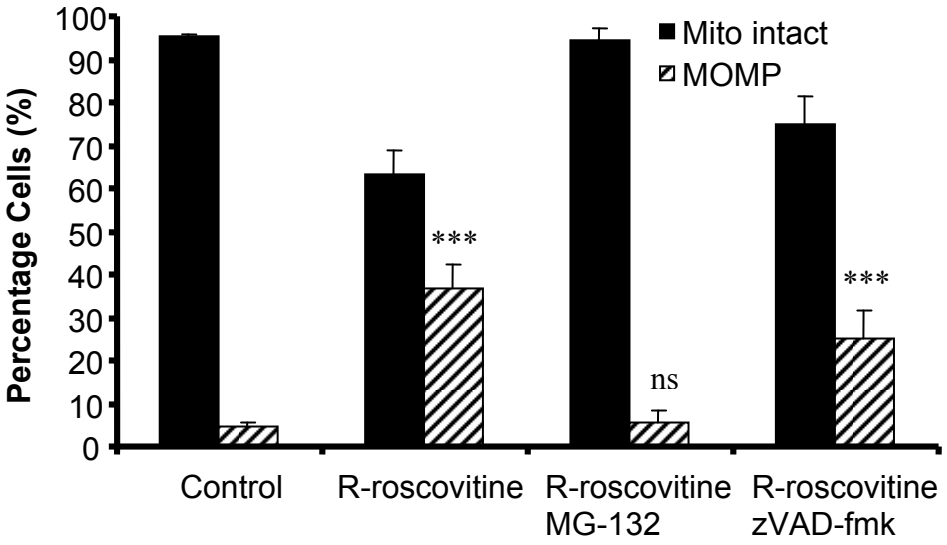


*Figure 4.4: Neutrophils were incubated with medium alone (Control) or+ R-roscovitine (20μM) alone or combined with MG-132 (50μM) or zVAD-fmk (100μM) for 2h and treated with mitocapture dye according to manufacturer's instructions (30min). The dye fluoresces red/orange in the mitochondria of viable neutrophils and green in the cytoplasm of neutrophils where there has been a shift in mitochondrial transmembrane potential. Fluorescence microscopy. All experiments (n=3). Statistically significant difference compared to control shown as ***, p<0.001 by ANOVA with a Student Newman-Keuls multiple comparison post hoc test with a 95% confidence interval.*

4.3 XIAP is not convincingly down-regulated by CDK inhibitor drugs

Given the known importance of the NF-κB pathway to neutrophil survival assessment of a survival protein known to be directly regulated by NF-κB was carried out. XIAP is known to inhibit activation of caspases 3, 7 and 9 (Jost et al. 2009). It has recently been suggested that XIAP may be the critical discriminator between type I (e.g., lymphocytes) and II (e.g., granulocytes) apoptosis (Jost et al. 2009). It has also been implicated in neutrophil survival mediated by G-CSF (van Raam et al. 2008) and is degraded in oxidant-induced neutrophil apoptosis (Gardai et al. 2004). Unfortunately, no consistent results were achieved immunoblotting for XIAP, perhaps because XIAP acts in the final stages of apoptosis and results are confounded by the conclusion of this process. It seems that in our system down-regulation of XIAP is more consistent with concluded apoptosis. PCR for the *XIAP* gene demonstrated no significant change in gene expression over the treatments studied (figure 4.5) a finding that was backed up by illumina gene chip technology.

Figure 4.5: *XIAP* is not significantly down-regulated at gene level

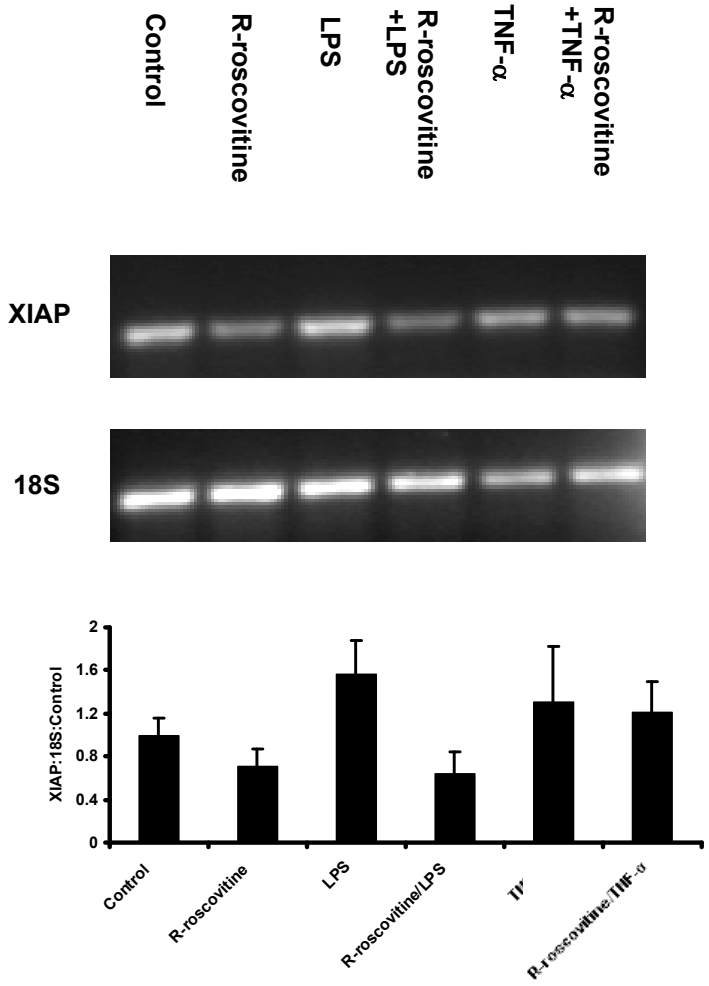


Figure 4.5: RNA extracted from neutrophils treated for 4h with R-roscovitine (20μM), LPS (100ng/ml), TNF-α (10ng/ml) or combination. Gels shown are representative and densitometry is from 3 experiments. No statistically significant difference for xiap between R-roscovitine and control, LPS and R-roscovitine/LPS, TNF-α and R-roscovitine/TNF-α (ANOVA with a Student Newman-Keuls multiple comparison post hoc test with a 95% confidence interval).

4.4 Effect of CDK inhibitor drugs on pro-apoptotic Bcl-2 homologues

In order to support the hypothesis that Mcl-1 is essential to neutrophil survival and that its down-regulation by CDK inhibitor drugs leads to apoptosis it was important to show that pro-apoptotic bcl-2 homologues were not down-regulated in a similar fashion. Mcl-1 prevents apoptosis by sequestering or otherwise blocking the ability of pro-apoptotic bcl-2 homologues to form pores in the outer mitochondrial membrane leading to intrinsic pathway apoptosis via the apoptosome. There has been strong evidence recently that the pro-apoptotic bcl-2 homologue Bim is prominent in neutrophil apoptosis and that it is especially relevant to apoptosis of cytokine-stimulated (i.e., akin to the inflammatory micro-environment) neutrophils (Andina et al. 2009;Cowburn et al. 2010). Other bcl-2 homologues previously identified to be of relevance to neutrophil apoptosis include Bax, Bid and Bad. We have already assessed Bid by western blotting (figure 3.13) and have an indirect indication of a predominance of pro-apoptotic bcl-2 homologues from measured loss (presumably facilitated by pro-apoptotic bcl-2 homologues) of mitochondrial potential (figure 4.4). Western blotting findings were supported by evidence from the use of a proteome profiler kit (figure 4.6) which seemed to indicate significant down-regulation of Bad and Bax but demonstrated no significant down-regulation of other pro-apoptotic bcl-2 homologues. In addition we have shown that Bim protein is preserved throughout a 4h treatment with the CDK inhibitor drug, R-roscovitine (Leitch et al. 2010b). We performed an illumina gene array for reasons which will be discussed in more depth in a later chapter and noted that at gene expression level *Bim* was significantly upregulated while *Bax* was significantly down-regulated and other pro-apoptotic bcl-2 homologues remained unaffected (figure 4.7).

Figure 4.6: Expression of various apoptosis-regulating proteins at protein level in neutrophil granulocytes treated with R-roscovitine

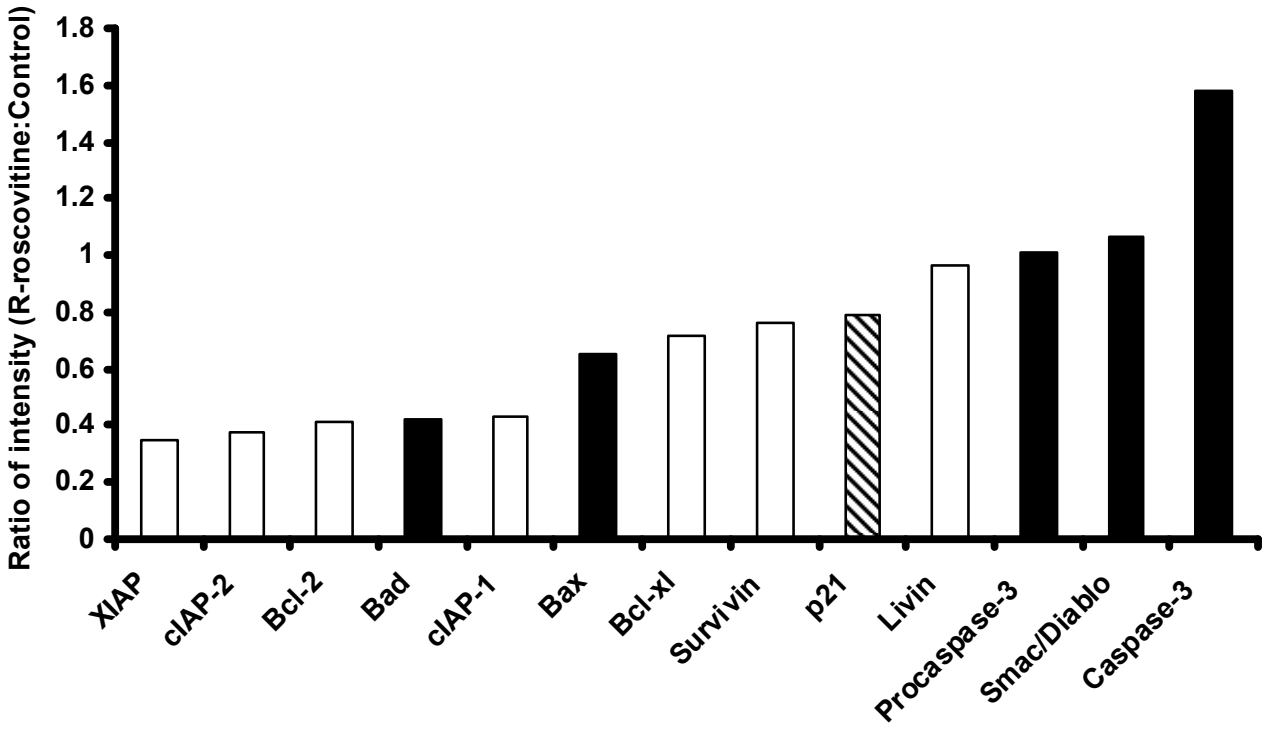


Figure 4.6: Neutrophils were prepared and treated as described previously for 4h with medium alone (control) or R-roscovitine (20μM). Cells were lysed according to manufacturer's (R&D) instructions and lysates were exposed to proteome profiler array membrane. Bespoke antibodies to apoptosis proteins shown above (most relevant to current thesis shown) pre-spotted onto array and streptavidin-HRP, ECL detection system used for quantification. Ratio of intensity was calculated by comparing R-roscovitine to Control having adusted for background intensity with the use of photoshop software (adobe). No statistics shown as n=1 with 2 replicates. Empty bars denote anti-apoptotic proteins, black bars are pro-apoptotic proteins and the stripedd bar is an endogenous CDK inhibitor important to apoptosis regulation.

Figure 4.7: Gene expression fold change of pro-apoptotic bcl-2 homologue proteins

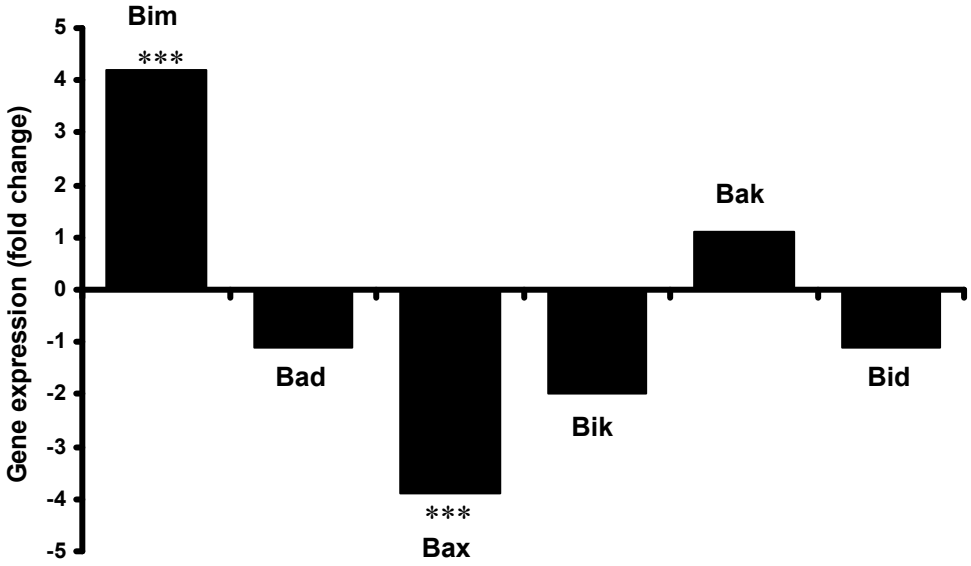


Figure 4.7: Effect of CDK inhibitor drugs on gene expression of pro-apoptotic bcl-2 homologues measured by Illumina Gene Chip. Neutrophils treated for 4h with media alone, or R-roscovitine (20μM), were subjected to RNA isolation using Nucleospin kit and Trizol extraction prior to interrogation by microarray. Result shows fold change in gene expression with statistical significance p<0.001 shown by ***.

4.5 Mcl-1 is down-regulated at the mRNA level

We hypothesized that R-roscovitine was mediating its effects on neutrophil survival by influencing production of key survival proteins at the level of transcription. We have confirmed this finding with the use of semi-quantitative PCR. R-roscovitine down-regulates *Mcl-1* at the mRNA level even in the presence of survival factors such as LPS and TNF-α (figure 4.8). By contrast, R-roscovitine did not significantly affect basal levels of *XIAP* mRNA at 4h but did seem to prevent its upregulation by LPS (not quite statistically significant when all samples analysed as per methods). TNF-α didn't appear to upregulate transcription of *XIAP*. Illumina gene array findings confirmed >2 fold down-regulation of Mcl-1 gene expression after treatment with R-roscovitine even in the presence of the survival factor LPS.

Figure 4.8: R-roscovitine down-regulates Mcl-1 at the level of mRNA

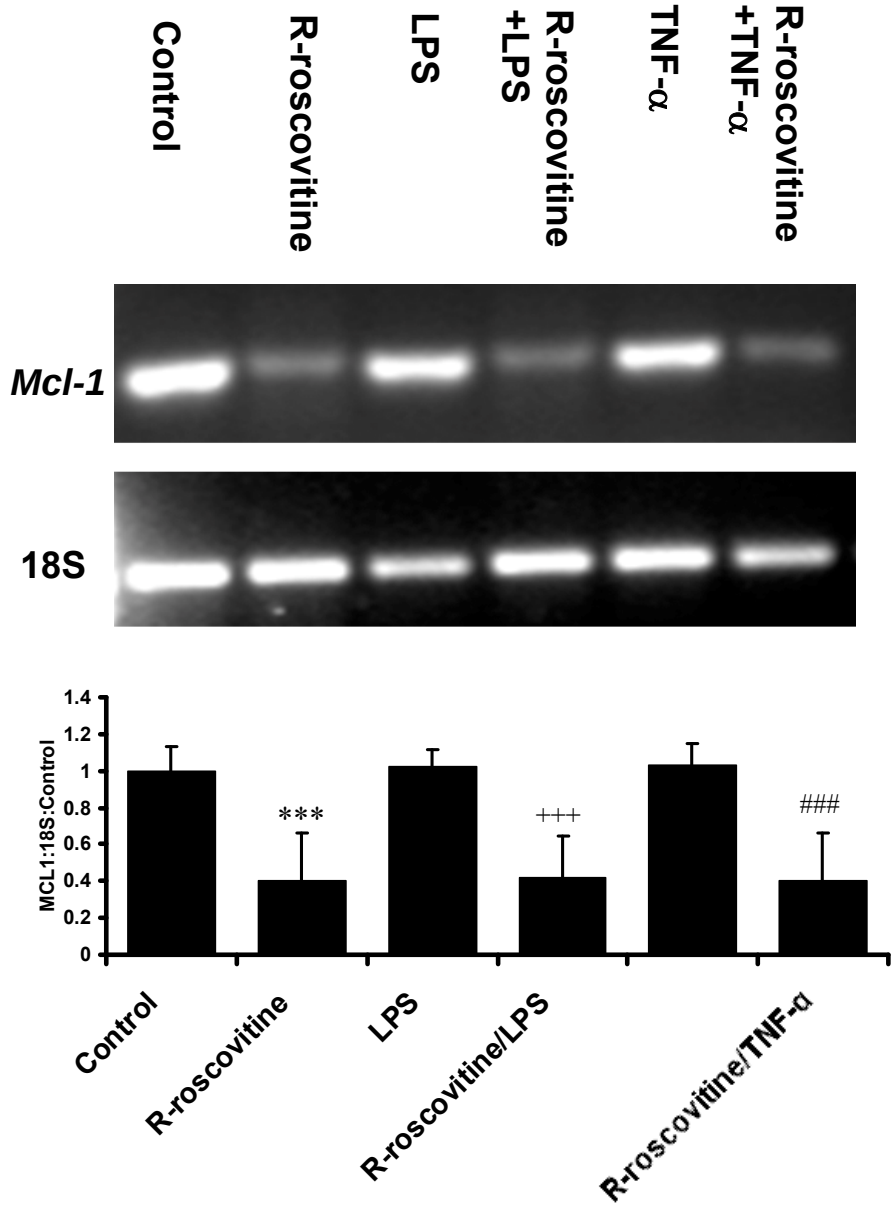


Figure 4.8: RNA extracted from neutrophils treated for 2h with R-roscovitine (20μM), LPS (100ng/ml), TNF-α (10ng/ml) or combination. Gels shown are representative and densitometry is from 3 experiments. Statistically significant difference for mcl-1 between R-roscovitine and control (***), LPS and R-roscovitine/LPS (+++) and TNF-α and R-roscovitine/TNF-α $p<0.001$ (###) (ANOVA with a Student Newman-Keuls multiple comparison post hoc test with a 95% confidence interval).

4.6 Discussion

The data presented in Chapter 4 pointed towards a key, short-lived neutrophil survival protein being preferentially down-regulated, possibly at a transcriptional level. Mcl-1 has already been postulated to be the most important survival protein produced by neutrophils and there is work suggesting that it is indispensable (Dzhagalov et al. 2007a;Edwards et al. 2004b). Mcl-1 down-regulation has been implicated in the induction of apoptosis by sodium salicylate (Derouet et al. 2006) and TNF-α (Cross et al. 2008) in neutrophil granulocytes and by dexamethasone (Meagher et al. 1996;Sivertson et al. 2007) in eosinophil granulocytes. By contrast, stabilisation of Mcl-1 is implicated in the anti-apoptotic effects of cAMP (Kato et al. 2006), GM-CSF (Derouet et al. 2004) and dexamethasone in neutrophils (Meagher et al. 1996;Sivertson et al. 2007). In addition Mcl-1 stabilisation is regarded as a key mechanism conferring aberrant survival on multiple myeloma cells(Le et al. 2004). We have previously shown that the CDK inhibitor R-roscovitine down-regulates Mcl-1 in eosinophil granulocytes to promote apoptosis (Duffin et al. 2009). Mcl-1 is increasingly recognised as indispensable to neutrophils as a result of the neutropenia evident in the myeloid-specific *mcl-1* knockout mouse (Dzhagalov et al. 2007a). Interestingly, macrophage/monocyte cells had normal longevity in this mouse. Mcl-1 is likely to play an important role in macrophage function however as it has been implicated in the successful resolution of pneumococcal pneumonia in mouse models (Marriott et al. 2005). Further support for the importance of Mcl-1 to neutrophil survival is given by work from two independent groups that have achieved antisense knockdown of Mcl-1 that induces neutrophil apoptosis (Leuenroth et al. 2000b;Sivertson et al. 2007). This effect was shown to overcome the Mcl-1 stabilising effects of dexamethasone and hypoxia.

In the preceding chapter I have attempted by a variety of approaches to demonstrate that down-regulation of Mcl-1 at a transcriptional level is central to the pro-apoptotic effects of R-roscovitine. Initially, I have demonstrated that Mcl-1 is down-regulated at the protein level in the presence of survival agents known to be present at sites of

inflammation including GMCSF, LPS and TNF-α (figure 4.1i). This is consistent with the finding from Chapter 1 that neutrophil apoptosis was promoted even in the presence of these same mediators. I have then shown that Mcl-1 can be preserved at protein level by co-incubation of neutrophils with the proteasome inhibitor MG-132 an effect that isn't evident with the caspase inhibitor z-VAD-fmk (figure 4.1ii).

MG-132 has previously been used as a surrogate NF-κB inhibitor because IκBα must be degraded in the proteasome (Ward et al. 1999). It follows therefore that where IκBα cannot be degraded it must persist and maintain inhibition of NFκB. This is not what might be regarded as a specific effect but was shown to have an impact on neutrophil longevity consistent with the hypothesized mechanism of action (at least at late time-points). However, it is also known that inhibition of the proteasome prevents degradation of the survival protein Mcl-1 and might be expected therefore to have a directly opposing effect (Derouet et al. 2006). Of course, it is within the bounds of reason that both effects might occur and be observed at different time-points consistent with the half-lives of the relevant proteins and the relevant kinetics of transcription and alternative methods of degradation attributed to the proteins. I have found that MG-132 preserves Mcl-1 at the protein level and that this effect translates into enhanced neutrophil survival in the presence of CDK inhibitor drugs (figure 4.2). This effect is time-limited, so that MG-132 must be administered to CDK inhibitor drug-treated neutrophils within 2h whilst the caspase inhibitor zVAD-fmk still confers some survival at 4h and 6h administration time-points (figure 4.3). The survival effect persists to 12h at which point rapid apoptosis occurs until viability levels are comparable to cells incubated with R-roscovitine alone by 20h (figure 4.2). Stabilisation of Mcl-1 by proteasome inhibition enhances neutrophil survival in the presence of CDK inhibitor drugs in a time-dependent fashion and preserves mitochondrial transmembrane potential (figures 4.2 and 4.4). It is difficult to infer what is ultimately responsible for the eventual loss of this survival effect but a number of hypotheses may be considered: 1.It is possible that proteasome inhibition is time-limited and that Mcl-1 eventually undergoes rapid degradation 2. NFκB inhibition may over-ride effects of Mcl-1 preservation 3.

Mcl-1 may be degraded by other means at this point (e.g. mediated by caspases) 4. Pro-apoptotic signalling may over-ride limited ability of neutrophil to synthesise more Mcl-1.

It was important to examine the expression of other pro-apoptotic and anti-apoptotic proteins in order to present a comprehensive picture of apoptosis signalling within the CDK inhibitor treated neutrophil. I used the proteome profiler kit to examine a wide-range of apoptosis implicated proteins in neutrophils untreated/treated with R-roscovitine (figure 4.6). This kit indicated that the general trend was for R-roscovitine to promote down-regulation of apoptosis-related proteins regardless of function. In fact the only significantly up-regulated protein was the executioner caspase, caspase-3. This result is consistent with a mechanism of action that targets transcription as opposed to survival signalling more directly. The findings in figure 4.6 were consistent with those at the gene expression level where down-regulation was again the general rule (figures 4.7 and 4.8). The exception to this rule was the pro-apoptotic protein Bim which was upregulated at gene expression level (figure 4.7) and appeared to maintain protein level (see paper appended, western blot performed by T. Sheldrake)(Leitch et al. 2010b) despite R-roscovitine treatment. Bim has come to the fore as the key pro-apoptotic bcl-2 homologue mediating neutrophil apoptosis (Andina et al. 2009;Cowburn et al. 2010). The *Bim* knockout mouse phenotype was of enhanced neutrophil survival (Bouillet et al. 1999) and two recent papers have shown Bim upregulation in neutrophils in models of inflammation *in vitro* and *in vivo* (Andina et al. 2009;Cowburn et al. 2010). Our finding is consistent with a critical excess of pro-apoptotic bcl-2 homologues occurring following down-regulation of survival proteins (especially Mcl-1) by CDK inhibitor drugs and the net result being loss of mitochondrial membrane integrity and apoptosis.
We have shown that Mcl-1 is down-regulated at the mRNA level by R-roscovitine and that this change is readily detected at 2h and is more pronounced at 4h (figure 4.8). We believe that Mcl-1 down-regulation precedes and is responsible for the initiation of apoptosis because we detect changes in the level of this mRNA/protein at 2h but only detect apoptosis by annexin-V binding at 4h and caspase cleavage at 2.5-3h (western

blotting and fluorometric caspase assay). By contrast R-roscovitine does not appear to decrease basal levels of *XIAP* but these data are not as clear-cut as the Mcl-1 findings, possibly because the half-life of *XIAP* mRNA is significantly longer than that of *Mcl-1* (Sharova et al. 2009;van Raam et al. 2008). The interpretation of results for TNF-α is complicated but interesting because of the dual and directly opposing effects of TNF-α on neutrophil apoptosis (Cross et al. 2008;Murray et al. 1997;Murray et al. 2003). TNF-α both directly activates the extrinsic apoptotic program and enhances transcription of key survival proteins via the NF-κB pathway. The combination of R-roscovitine and TNF-α leads to very early initiation of and bias towards the extrinsic apoptotic program detectable by annexin-V binding at 2h (data not shown). XIAP is upregulated and stabilised in response to caspase cleavage so it might be expected that we would find enhanced mRNA/protein levels of XIAP. However, there is no detectable upregulation as measured by PCR which probably represents a degree of upregulation in response to the early initiation of apoptosis that is balanced by inhibition of transcription by R-roscovitine. We have shown that *de novo* transcription of XIAP as stimulated by LPS is prevented by R-roscovitine.

By demonstrating that the important survival protein Mcl-1 is down-regulated at the level of transcription (figure 4.8), we corroborate our finding that direct inhibition of NF-κB is not responsible for CDK inhibitor driven neutrophil apoptosis (Leitch et al. 2010b). We hypothesise that a more central, generalised effect on neutrophil transcription is responsible for the induction of apoptosis. This effect is particularly evident in granulocytes because of the importance of rapidly transcribed and degraded, short-lived protein Mcl-1 to their survival. Pro-apoptotic bcl-2 homologues have much longer half-lives and in excess drive neutrophil apoptosis via the intrinsic (mitochondrial) pathway. In addition the pro-apoptotic bcl-2 homologue Bim appears to be upregulated at gene level in response to CDK inhibitor drug treatment. Our work adds to the literature suggesting the fundamental importance of these proteins to neutrophil

survival and further illuminates the mechanism of action of the CDK inhibitor drug, R-roscovitine.

4.7 Summary of Chapter 4

1. **The key neutrophil survival protein, Mcl-1, is down-regulated by CDK inhibitor drugs** *as assessed* by western blotting
2. **Inhibition of the proteasome prevents Mcl-1 degradation and delays CDK inhibitor drug induced apoptosis** *as assessed by western blotting and flow cytometry for annexin-V/PI*
3. **Mcl-1 is down-regulated at the level of transcription** *as assessed by semi-quantitative PCR and illumina gene-chip technology*
4. **Pro-apoptotic bcl-2 homologue Bim is up-regulated by CDK inhibitor drugs** *as assessed by western blotting and illumina gene chip technology*

5. Results: CDKs and cyclins are present in neutrophils

The presence, distribution and functionality of CDKs, their cyclin binding partners and endogenous inhibitors has not previously been subjected to detailed analysis in neutrophils. It has presumably been felt that any detection of these proteins would merely be the cataloguing of vestigial remnants of the differentiation process from myeloid precursors. With the discovery that inhibitors of CDKs can significantly influence cell viability it may be inferred that these kinases contribute something important if not irreplaceable to terminally differentiated neutrophils. I have already discussed at some length in the introduction the different functionalities of CDKs in other cells and cell-lines. In this chapter I intend to establish the existence of key CDKs, cyclins and endogenous inhibitors in neutrophils, to document their responsiveness to known neutrophil survival factors and to investigate the impact of CDK inhibitor drugs. This will be achieved by a combination of western blotting with differential nuclear/cytoplasmic lysis, illumina gene chip technology and confocal microscopy. The function of the key CDKs 7 and 9 is assessed by western blotting for phosphorylation of the transcription enzyme RNA pol II.

5.1 Identification of CDKs and cyclins at protein level in human neutrophils

CDKs were identified at protein level by western blotting (figure 5.1). Western blotting of nuclear and cytoplasmic lysates from neutrophils was performed to investigate the sub-cellular distribution of CDK proteins. We initially concentrated on CDKs known to be within the specificity of our nominated inhibitor R-roscovitine (i.e., CDKs 2,5,7 and 9).

CDK2 was difficult to detect and despite attempts with two different primary antibodies at a range of primary and secondary antibody concentrations with various blocking

solutions it was only detectable as a faint band on prolonged exposure. This is in keeping with previous findings from our group (Rossi et al. 2006). CDK2 appeared to be largely cytoplasmic in distribution although there is some detected in the nuclear fraction when cells are stimulated with LPS (figure 5.1).

CDK5 was easily detected and was distributed between the cytoplasmic and nuclear fractions with the cytoplasmic fraction predominating. There appears to be some increase in the nuclear fraction of CDK5 or perhaps just an increase in total CDK5 protein on stimulation with LPS that is blocked by co-treatment with R-roscovitine (figure 5.1).

CDK7 was again easily detected and in a similar distribution to CDK5 though R-roscovitine appeared to have less impact on the ability of LPS to upregulate protein levels generally and/or to enhance nuclear translocation (figure 5.1).

Two distinct isoforms of CDK9 have previously been described in the literature and both of these were easily detected for the first time in neutrophils. The 55kDa isoform was equally distributed between nuclear and cytoplasmic fractions while the 43kDa isoform appeared predominantly in the nuclear fraction. There was no obvious effect of LPS stimulation or R-roscovitine treatment (figure 5.1).

The binding partner cyclins of CDKs 7 and 9 (cyclin H and cyclin T_1) were identified as these CDKs seemed targets of particular interest from the published literature with regard to R-roscovitine. The cyclin proteins were predominantly expressed in the nuclear fraction which is consistent with the published literature. There appeared to be minimal impact of LPS stimulation or R-roscovitine treatment (figure 5.1).

Figure 5.1: Presence of selected CDKs and cyclin binding partners in neutrophil granulocytes and response to stimulation and CDK inhibitor drugs

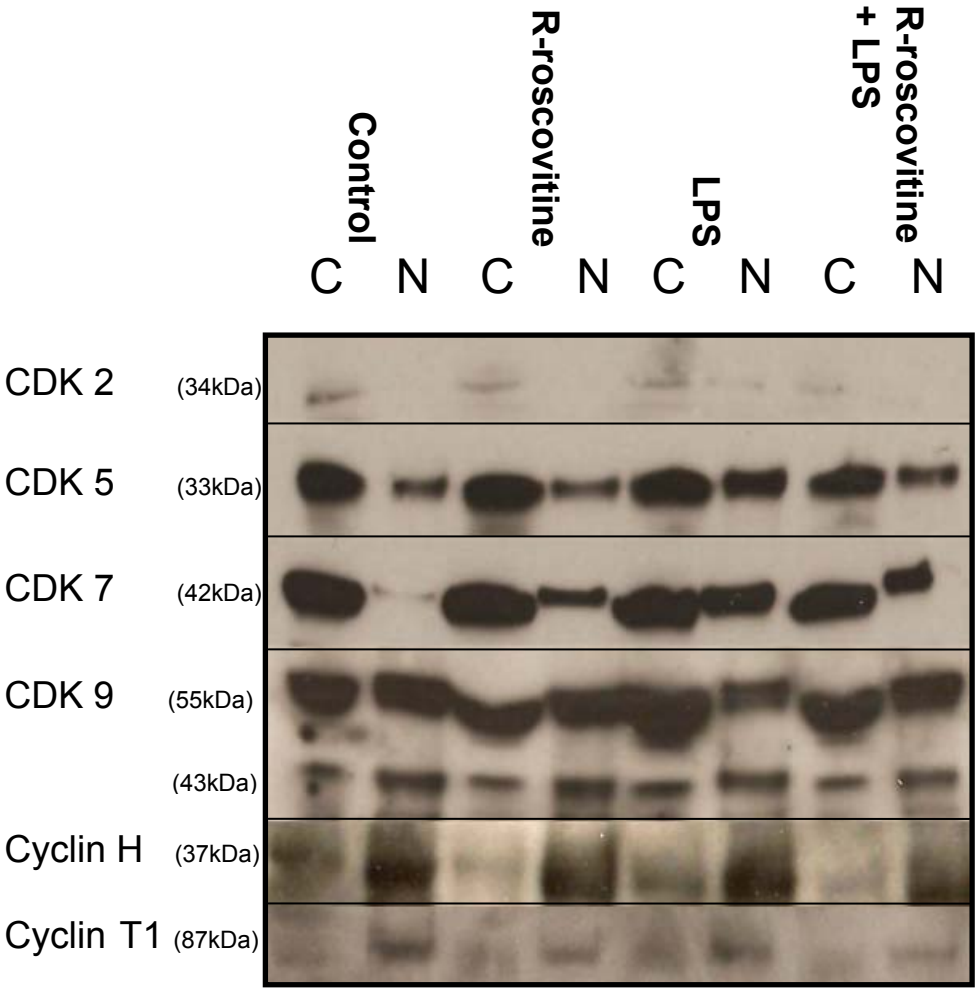


Figure 5.1: Western blotting for CDKs known to be inhibited by R-roscovitine and cyclin binding partners of CDKs 7 and 9. Neutrophils were incubated as previously indicated for western blotting with agents as indicated above at the following concentrations: R-roscovitine 20μM, LPS 100ng/ml. At 4h cells were lysed using neutrophil nuclear/cytoplasmic lysis buffers and technique (see methods). Lysates were run on 10% pre-cast gels and protein identified by marker analysis. All gels are representative of n=3 experiments.C=Cytoplasmic fraction , N=Nuclear fraction.

In order to confirm the data accumulated by western blotting confocal microscopy of neutrophils labelled by indirect immunofluorescence was performed (figures 5.2 and 5.3). Freshly isolated neutrophils were transferred onto glass cover-slips, fixed, permeabilised and labelled with the primary antibody of interest. Secondary fluorescent antibodies were alexa-fluor^{TM} with an excitation maximum of 647nm (far-red spectrum) to minimise the potential for interaction with a high degree of auto-fluorescence generated by granulocyte cells. Cells were also stained with DAPI to allow estimation of nuclear co-localisation.

CDKs 7 and 9 were identified within the nucleus and cytoplasm of neutrophils and confocal imaging indicated that CDK7 and was strongly expressed in the nucleus in a constitutive manner and appears in an inverse nuclear pattern to DAPI (figure 5.2). DAPI staining is greatest at the nuclear periphery as this is where chromatin is most condensed. CDK7 appears more centrally within the nucleus. It has been suggested that transcriptionally active chromatin is less condensed (Bartova et al. 2001;Bartova et al. 2002;Yerle-Bouissou et al. 2009). CDK9 appeared to be predominantly expressed in the cytoplasm and though there was evidence of nuclear co-localisation it was less striking than that observed with CDK7 (figure 5.3). It was difficult to reliably identify CDKs 2 and 5 by confocal microscopy despite careful investigation. This is in keeping with the western blotting data for CDK2 but not for CDK5 where protein was easily detected. The main problem with confocal microscopy for CDK5 was significant background and it may be that the gentler washing steps required to maintain cell integrity in confocal microscopy were not sufficient to discriminate between specific antibody binding and background.

In addition there were differences in the apparent levels of nuclear CDK as measured by western blotting and confocal microscopy. CDK7 appeared to be predominantly cytoplasmic by western blotting but nuclear by confocal microscopy. Western blotting, especially nuclear/cytoplasmic lysis is complicated in neutrophils by the excessive levels of proteases generated by these cells. There is great potential to lose protein despite careful handling. Although protein levels were assessed by BCA assay and cytoplasmic

fraction was diluted accordingly it is still possible that there was comparatively more protein loss in western blotting compared to confocal microscopy.

Figure 5.2: Confocal microscopy of CDK7 in neutrophil granulocytes

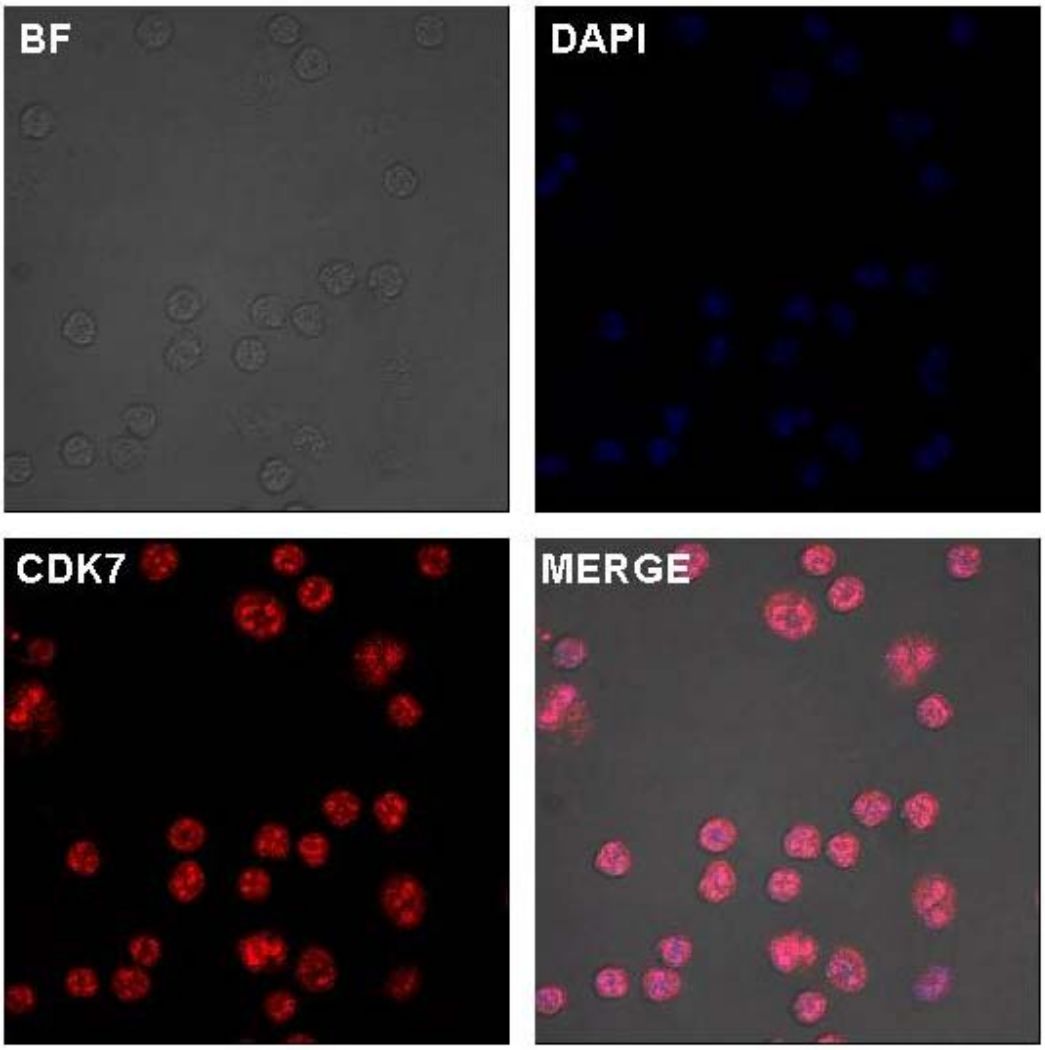


Figure 5.2: Confocal microscopy of CDK7 in neutrophils by indirect immunofluorescence. Upper left panel shows transmitted light/bright field (BF), upper right panel shows DAPI nuclear staining (blue), lower left panel shows CDK7 staining (red), lower right panel shows merged image (MERGE) demonstrating nuclear colocalisation (pink). Leica SP confocal microscope x630 oil immersion. Representative image of at least n=3 experiments.

Figure 5.3: Confocal microscopy of CDK9 by indirect immunofluorescence

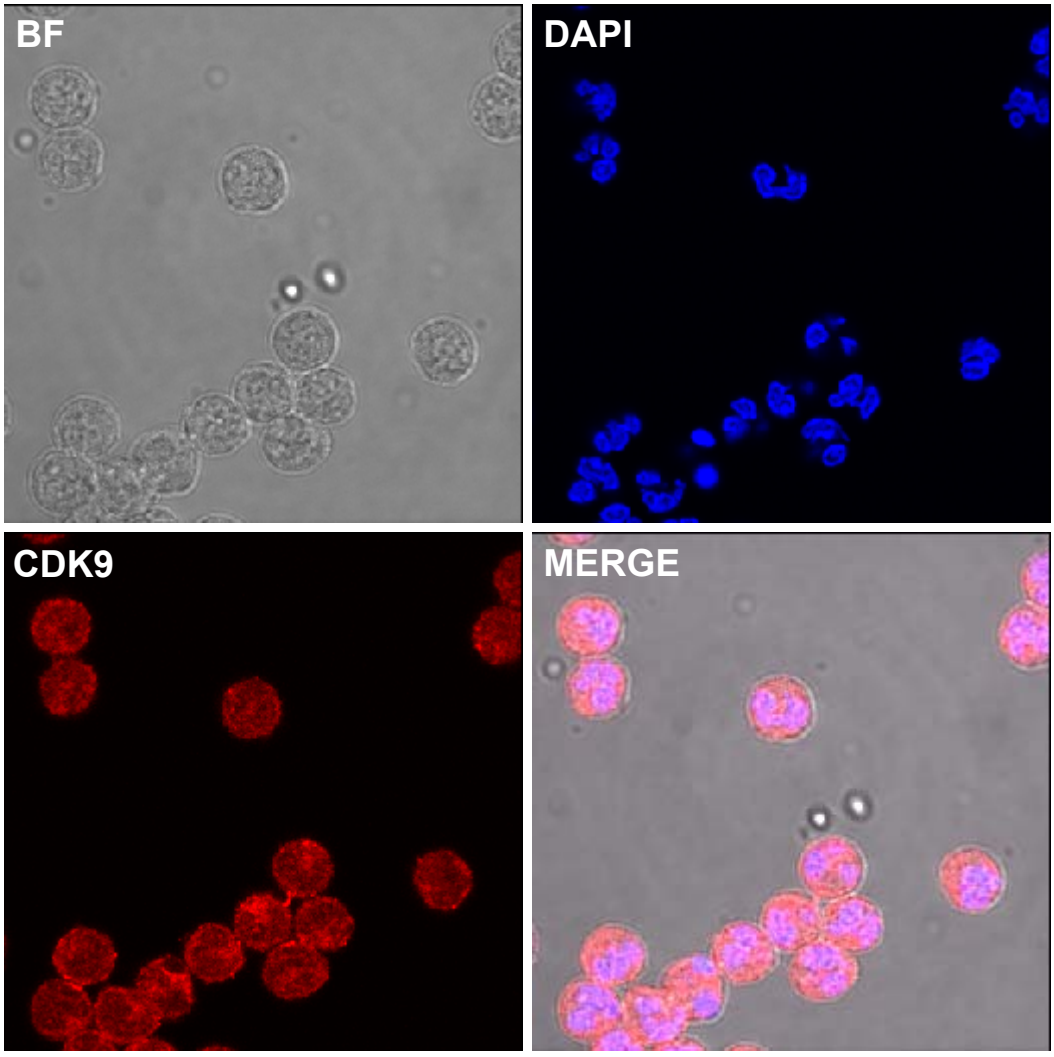


Figure 5.3: Confocal microscopy of CDK9 in neutrophils by indirect immunofluorescence. Upper left panel shows transmitted light/bright field (BF), upper right panel shows DAPI nuclear staining (blue), lower left panel shows CDK9 staining (red), lower right panel shows merged image (MERGE) demonstrating nuclear co-localisation (pink). Leica SP confocal microscope x630 oil immersion. Representative image of at least n=3 experiments.

5.2 Sub-cellular distribution of CDK7 and 9 in neutrophils following stimulation with LPS by confocal microscopy

In order to investigate a functional role for the sub-cellular distribution of CDKs in neutrophils I again utilised western blotting (figure 5.1) and confocal microscopy (figure 5.4 and 5.5) to detect changes in the sub-cellular localization of CDKs on stimulation with LPS and in the presence of CDK inhibitor drugs. Confocal microscopy for CDK7 in neutrophils stimulated with LPS allowed easier detection of the protein presumably because there was more CDK7 protein to detect in stimulated cells (figure 5.4). CDK9 appears in an inverse nuclear pattern to DAPI staining following LPS stimulation. (figure 5.5). It might be inferred that LPS stimulation drives recruitment of CDK9 to decondensed chromatin or that LPS stimulation drives chromatin decondensation allowing CDK9 recruitment. The sub-cellular distribution of CDKs 7 and 9 was unaffected by CDK inhibitor drug treatment though it was noticeable that CDK7 fluorescence was less intense following co-incubation of neutrophils with LPS and R-roscovitine compared to LPS alone and more akin to the intensity observed constituively (data not shown).

Figure 5.4: CDK7 staining of neutrophils after LPS stimulation

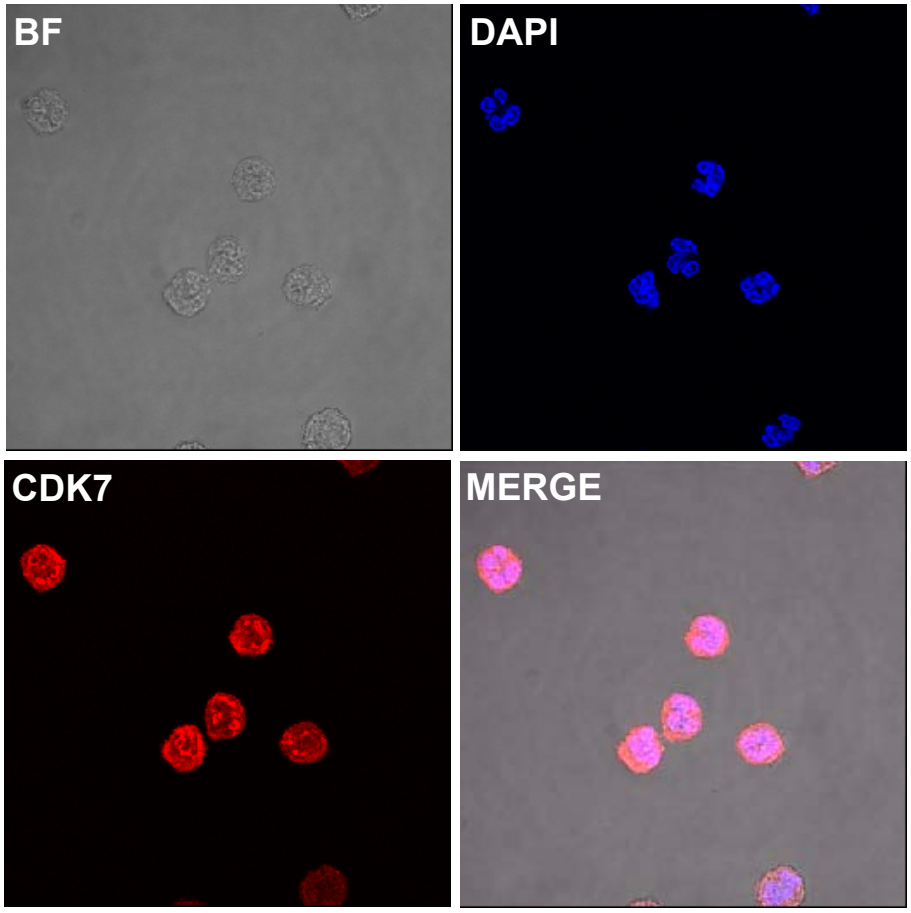


Figure 5.4: Confocal microscopy of neutrophils stimulated by LPS (100ng/ml) and probed for CDK7 by indirect immunofluorescence. Upper left panel shows transmitted light/bright field (BF), upper right panel shows DAPI nuclear staining (blue), lower left panel shows CDK7 staining (red), lower right panel shows merged image (MERGE) demonstrating nuclear colocalisation (pink). Leica SP confocal microscope x630 oil immersion. Representative image of at least n=3 experiments.

Figure 5.5: CDK9 staining of neutrophils after LPS stimulation

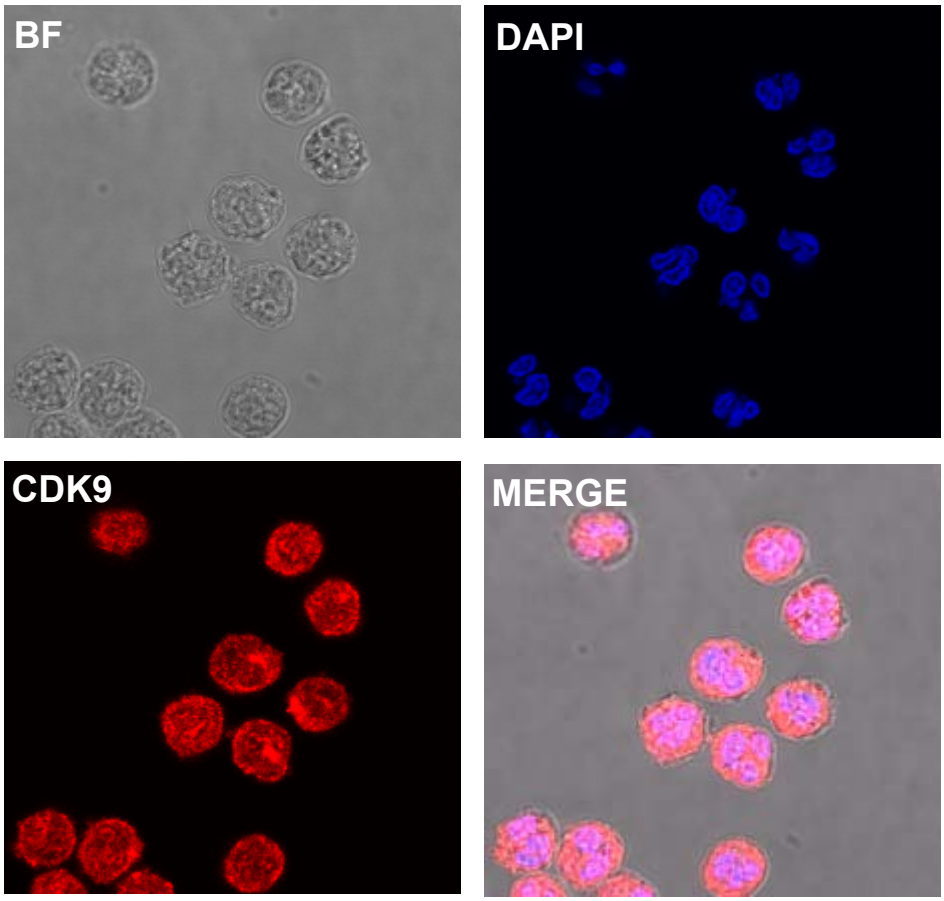


Figure 5.5: Confocal microscopy in neutrophils stimulated with LPS (100ng/ml) and probed for CDK9 by indirect immunofluorescence. Upper left panel shows transmitted light/bright field (BF), upper right panel shows DAPI nuclear staining (blue), lower left panel shows CDK9 staining (red), lower right panel shows merged image (MERGE) demonstrating nuclear co-localisation (pink). Leica SP confocal microscope x630 oil immersion. Representative image of at least n=3 experiments.

5.3 Identification of CDKs, endogenous CDK inhibitors and cyclins at gene level in human neutrophils

Using Illumina genechip technology we identified CDK gene expression levels in untreated/unstimulated neutrophils and compared this with neutrophils stimulated with LPS and either treated with R-roscovitine or not (figure 5.6). Neutrophils were incubated as previously described and purity of the cell population obtained by gradient method was checked by cytospin and flow cytometry. Granulocytes accounted for 99.3% of the cell population using this methodology and eosinophils less than 2% of the granulocyte population. Once neutrophil purity was confirmed RNA isolation was performed by Trizol extraction. RNA purity and quantification were initially performed with the use of a nanodrop 100. RNA was further quantified and assessed with the use of an Agilent Bioanalyser before the gene-chip assay was performed. The most strongly expressed *CDKs* appeared to be *CDKs 2, 7* and *9* (figure 5.6). Interestingly, there was an induction of gene expression of CDKs 2 and 7 in neutrophils stimulated with LPS (figure 5.6). This induction appeared to be inhibited by over 50% when LPS-stimulated cells were co-treated with R-roscovitine.

The cyclin binding partners of CDK7 and 9 (cyclin H and cyclin T1 respectively) were expressed at higher levels than other cyclins (*cyclin D1* shown for comparison) (figure 5.7). *Cyclin H* expression was negatively regulated to a statistically significant degree by R-roscovitine treatment. MAT1 another binding partner of CDK7 as part of the transcription factor complex TFIIH was expressed at low levels that were unaffected by treatment or stimulation.

Endogenous CDK inhibitors, p21 and p27 were significantly expressed at gene level by neutrophils and *p21 variant (v) 1* was upregulated by LPS treatment (figure 5.8). By comparison *p21v2* was only minimally expressed. *p21v1* is known to be regulated by LPS but it was interesting to note that R-roscovitine significantly blocked the ability of LPS to upregulate *p21* gene expression by approx. 30%.

Figure 5.6: CDK gene expression by neutrophil granulocytes

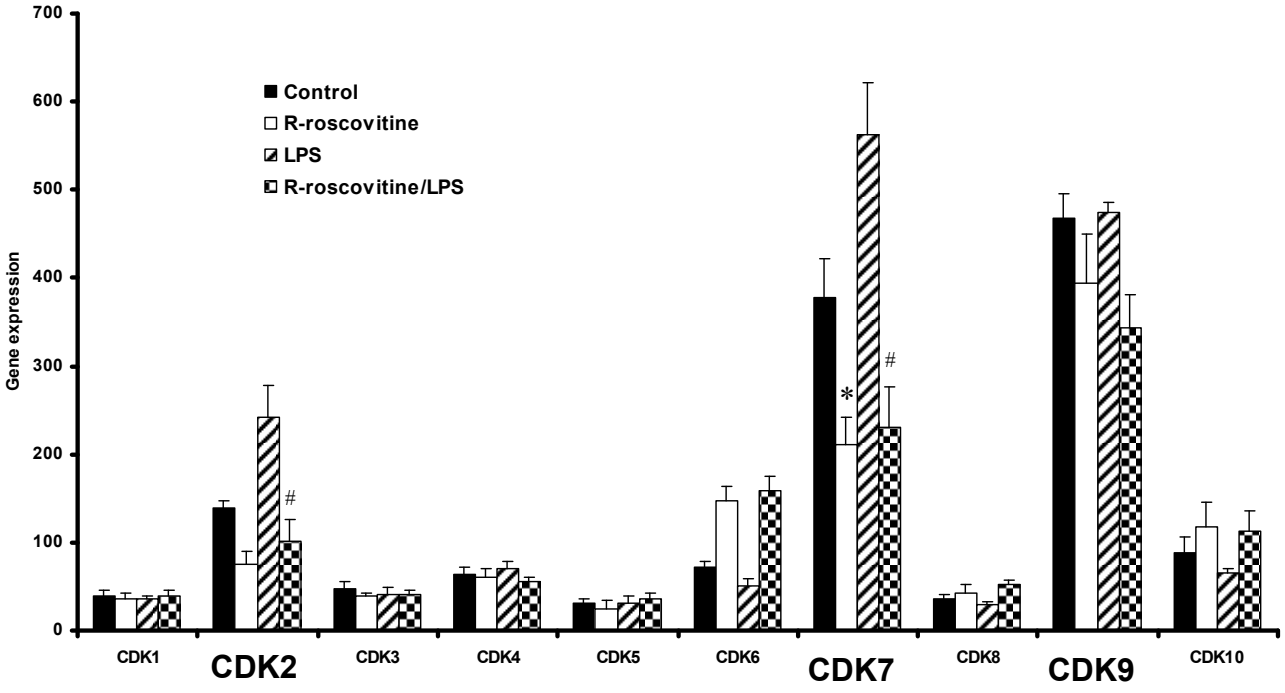


*Figure 5.6: Illumina Gene Chip expression levels of neutrophil CDKs. Neutrophils treated for 4h with media alone, R-roscovitine (20μM), LPS (100ng/ml) or a combination were subjected to RNA isolation using Nucleospin kit and Trizol extraction prior to interrogation by microarray. Result shows gene expression levels of CDK 1-10 genes as mean+/- SEM. Significant differences p<0.001 between Control and R-roscovitine are shown as * and between LPS and R=roscovitine/LPS as # by ANOVA with post hoc multivariate analysis by Student Newman-Keuls .*

Figure 5.7: Cyclin and binding partner gene expression in neutrophil granulocytes

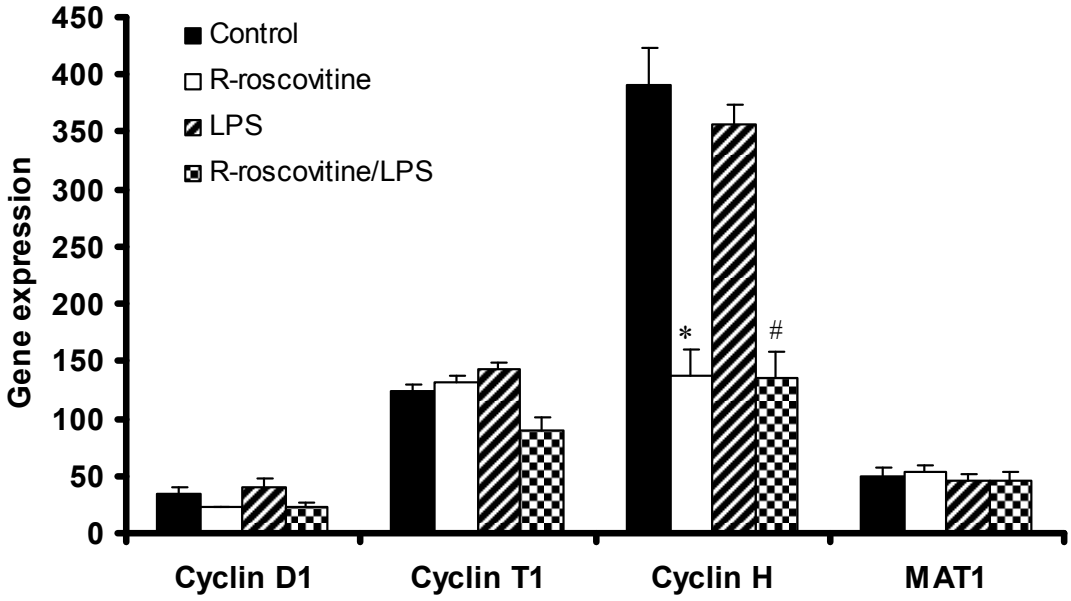


*Figure 5.7: Illumina Gene Chip expression levels of neutrophil cyclins and binding partners. Neutrophils treated for 4h with media alone, R-roscovitine (20μM), LPS (100ng/ml) or a combination were subjected to RNA isolation using Nucleospin kit and Trizol extraction prior to interrogation by microarray. Result shows gene expression levels of cyclin and MAT1 genes as mean+/- SEM. Significant differences p<0.001 between Control and R-roscovitine are shown as * and between LPS and R=roscovitine/LPS as # by ANOVA with post hoc multivariate analysis by Student Newman-Keuls.*

Figure 5.8: Endogenous CDK inhibitor gene expression in neutrophil granulocytes

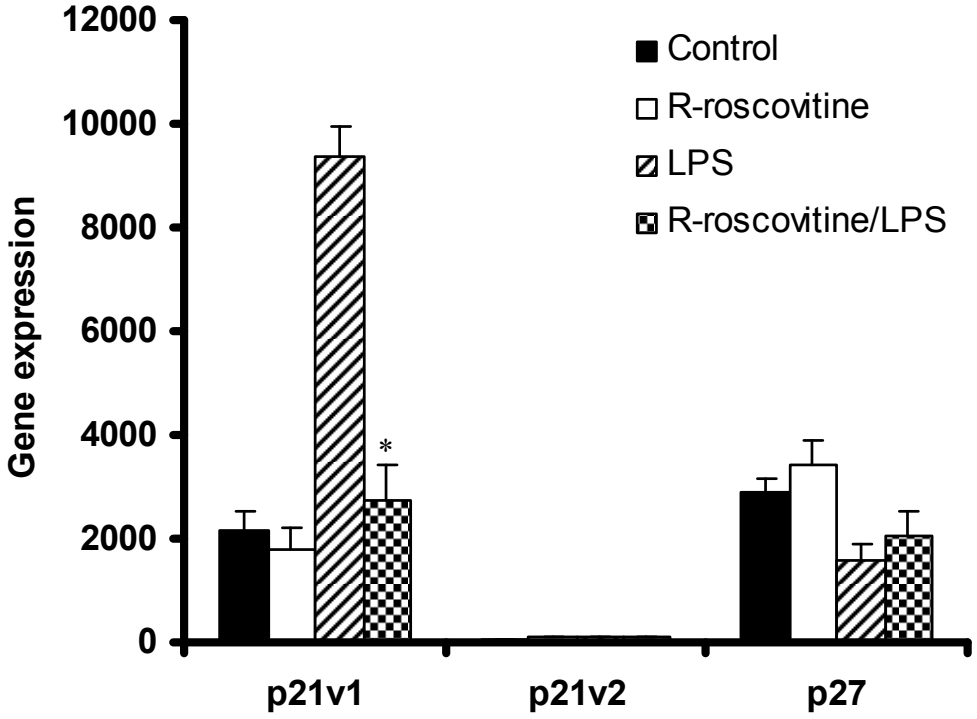


*Figure 5.8: Illumina Gene Chip expression levels of neutrophil endogenous CDK inhibitors. Neutrophils treated for 4h with media alone, R-roscovitine (20μM), LPS (100ng/ml) or a combination were subjected to RNA isolation using Nucleospin kit and Trizol extraction prior to interrogation by microarray. Result shows gene expression levels of endogenous CDK inhibitor genes as mean+/- SEM. Statistically Significant differences p<0.001 between LPS and Control shown as * by ANOVA with post hoc multivariate analysis by Student Newman-Keuls .*

5.4 RNA polymerase II is present in phosphorylated and unphosphorylated forms in neutrophils and the process of phosphorylation is modulated by CDK inhibitor drugs

It has previously been argued that neutrophils have no transcriptional capacity and that any RNA isolated from these cells is pre-packaged having been assembled earlier in the differentiation process. This theory has subsequently been discounted by a number of groups that have found evidence for *de novo* transcription in a variety of circumstances (Cassatella 1995;McDonald et al. 1998).

The aim of this experiment was to identify evidence for previously unidentified neutrophil transcriptional machinery and to examine evidence for the involvement of CDKs 7 and 9 in the regulation of transcription. Additionally, phosphorylation of the CTD of RNA pol II which consists of 52 repeats of heptapeptide Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 (YSPYSPS) provides a functional assay for CDK7 and 9 activity. CDK7 is known to phosphorylate RNA pol II serine 5 whilst CDK9 is known to phosphorylate predominantly serine 2 (though it can phosphorylate both under certain conditions in different cell types).

In figure 5.9 the total RNA polymerase protein complex (Poltot) and RNA polymerase phosphorylated on key serine residues at position 2 (PS2) and 5 (PS5) are identified by western blotting with densitometry quantification. Phosphorylation on these serine residues is of known importance to the regulation of transcription by CDKs 7 and 9. Significant time-dependent loss of phosphorylation at serine residues 2 and 5 when neutrophils are incubated with the CDK inhibitor R-roscovitine is shown (figure 5.9). In addition the RNA pol II protein is identified by indirect immunofluorescence detected by confocal microscopy (figure 5.10 and 5.11). Confocal microscopy reveals the inverse DAPI staining pattern previously seen with stimulated neutrophils stained for CDK7 and

9 and is consistent with RNA pol II being present at sites of active gene transcription. This pattern is enhanced in neutrophils stimulated with LPS.

Figure 5.9: RNA polymerase II phosphorylation in presence and absence of CDK inhibitor drugs

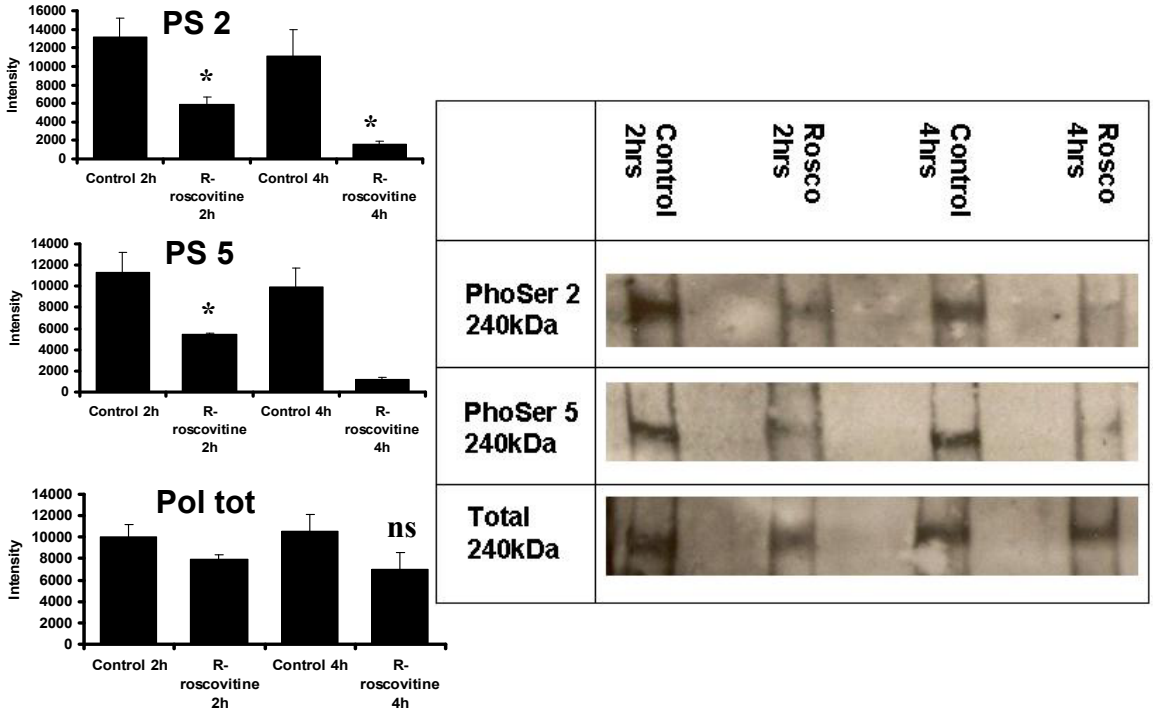


*Figure 5.9: Western blotting for RNA polymerase II total (Pol tot), phosphorylated on serine 2 (PS2) and phosphorylated on serine 5 (PS5). Neutrophils incubated as previously described with media (Control) or R-roscovitine (Rosco) (20μm). Nuclear/cytoplasmic lysis protocol was used to optimize protein retrieval and lysates were run on 4% acrylamide gels. Blots shown are representative of 3 experiments and densitometry is data from 3 experiments shown as mean +/- SEM. Statistical significance compared to relevant time-point control $p<0.001$ is shown as ** and $p<0.05$ * by ANOVA with post hoc multivariate analysis by Student Newman-Keuls with a 95% confidence interval).*

Figure 5.10: Confocal microscopy of neutrophils probed for RNA polymerase II (total) by indirect immunofluorescence

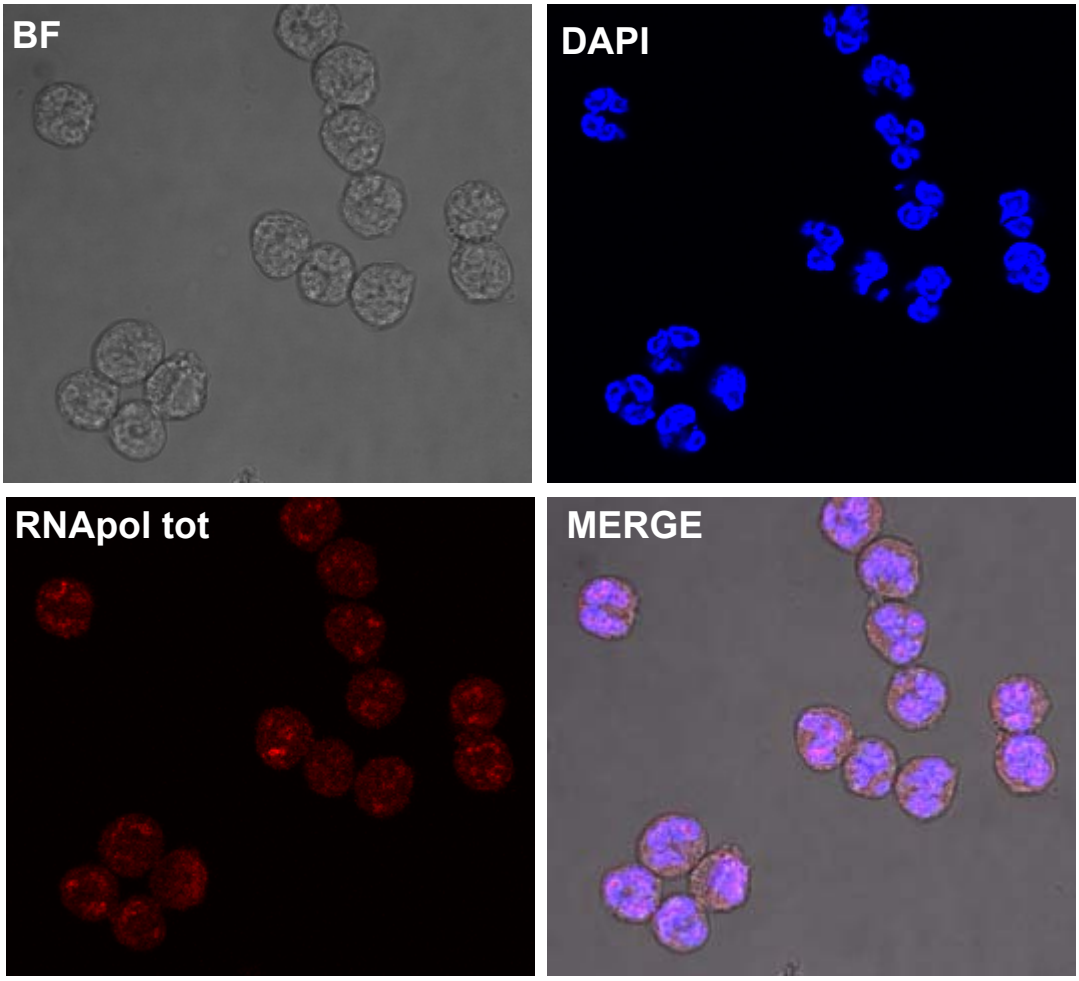


Figure 5.10: Confocal microscopy of RNA pol II (total) in neutrophils by indirect immunofluorescence. Upper left panel shows transmitted light/bright field (BF), upper right panel shows DAPI nuclear staining (blue), lower left panel shows RNA pol II (total) staining (red), lower right panel shows merged image (MERGE) demonstrating nuclear colocalisation (pink). Leica SP confocal microscope x630 oil immersion. Representative image of at least n=3 experiments.

Figure 5.11: Confocal microscopy of neutrophils stimulated with LPS and probed for total RNA polymerase II by indirect immunofluorescence

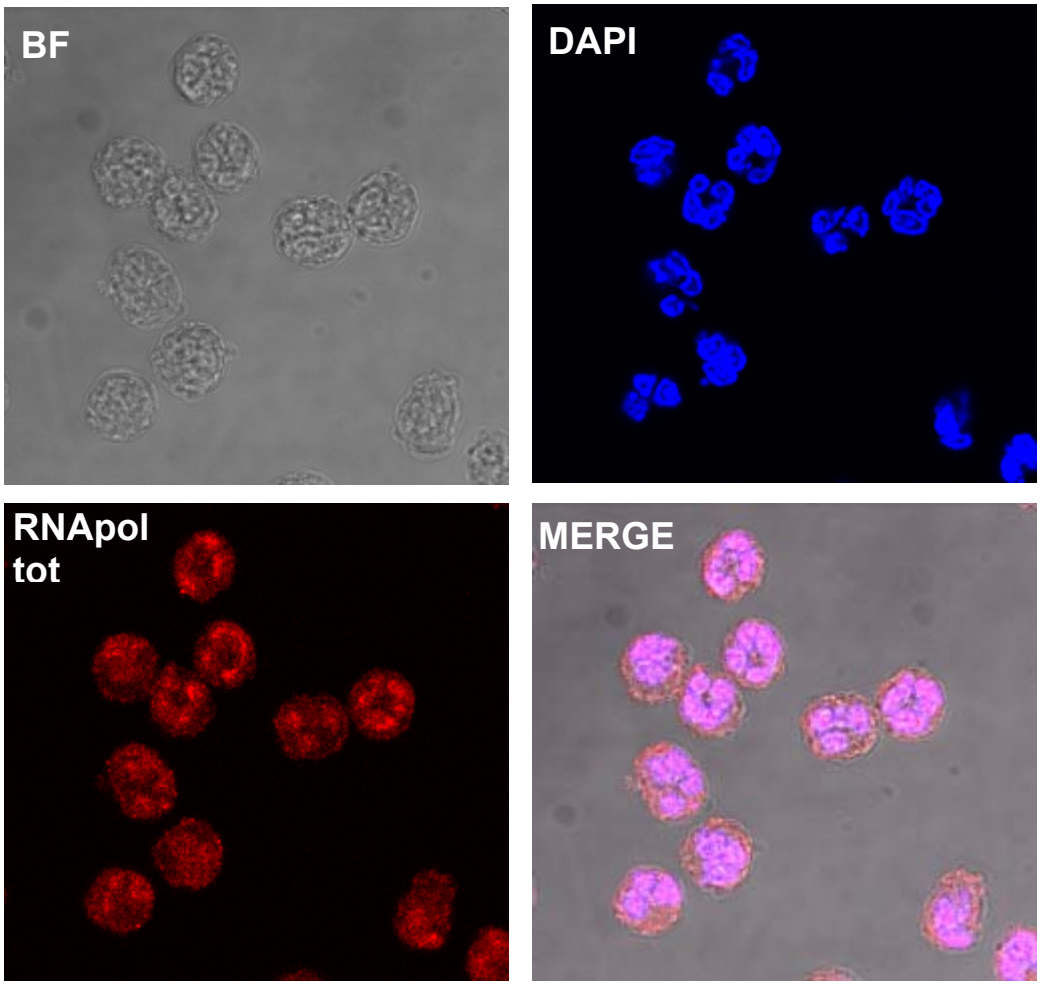


Figure 5.11: Confocal microscopy of neutrophils stimulate with LPS (100ng/ml) probed for RNA pol II (total) by indirect immunofluorescence. Upper left panel shows transmitted light/bright field (BF), upper right panel shows DAPI nuclear staining (blue), lower left panel shows total RNA pol II (RNApol tot) staining (red), lower right panel shows merged image (MERGE) demonstrating nuclear colocalisation (pink). Leica SP confocal microscope x630 oil immersion. Representative image of at least n=3 experiments.

5.5 Down-regulation of RNA polymerase II phosphorylation by CDKs 7 and 9 has specific effects on gene transcription

In figure 5.12 global effects on gene expression assessed by illumina gene chip technology for neutrophils treated with R-roscovitine are illustrated by representative bar-graphs and a heat map. The heat map shows the genes most significantly up- and down-regulated across all four treatment conditions (untreated neutrophils (C), R-roscovitine-treated (R), LPS-stimulated (L) and LPS/R-roscovitine co-treatment (RL)). In total of genes detected: 1656 genes were down-regulated, 886 were upregulated and 1359 weren't significantly affected following treatment with R-roscovitine for 4h. Interestingly, though perhaps not surprisingly genes related to transcription were highly represented in terms of differential regulation following R-roscovitine treatment. In figure 5.13 you can see the impact on transcription related genes as well as data for further gene types of specific interest to this thesis (apoptosis, inflammation, cell-cycle). The general trend is of down-regulation of genes. Using gene ontology cluster analysis (figure 5.14) it is possible to identify clusters of genes that are most affected by R-roscovitine treatment (in terms of both up- and down-regulation of expression). Those highlighted in bold are supportive of the hypotheses examined in this thesis.

Figure 5.12: Effect of CDK inhibitor drugs on the neutrophil transcriptome

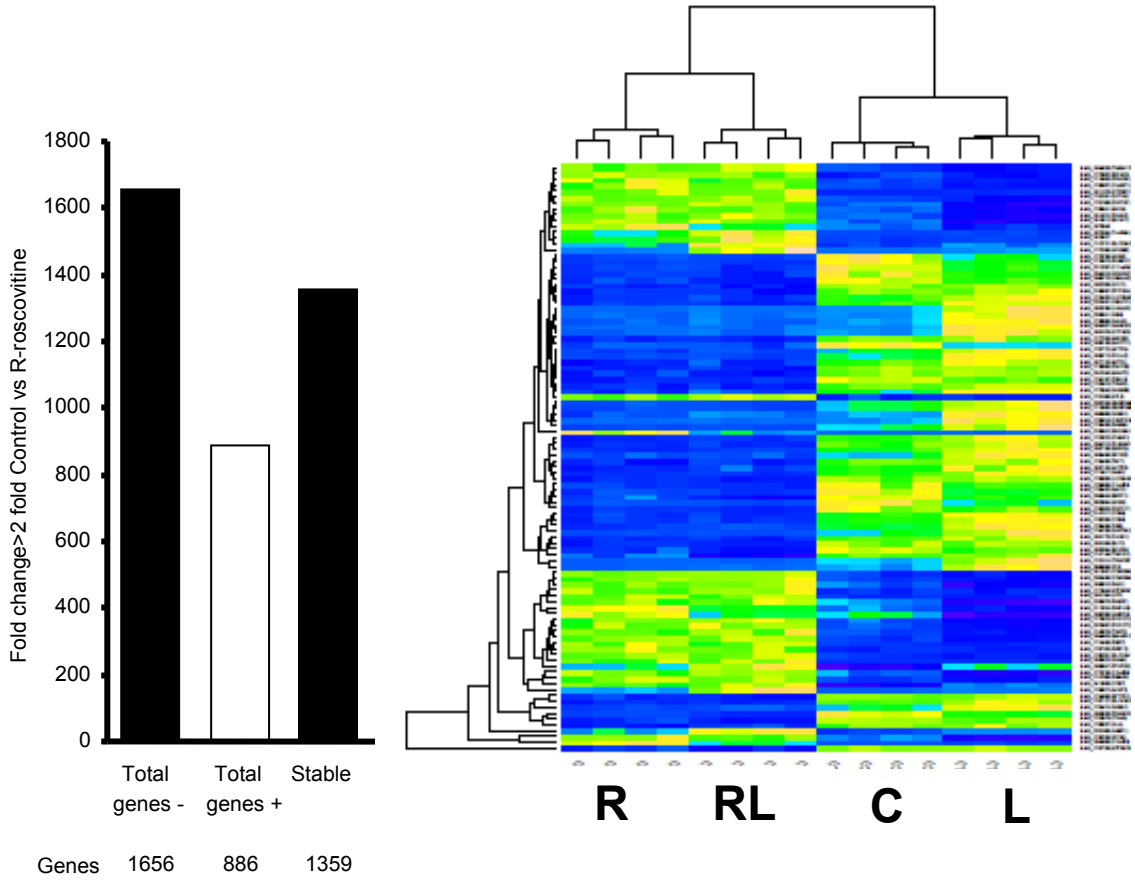


Figure 5.12: Illumina gene chip effect of CDK inhibitor drugs on the neutrophil transcriptome and specifically on genes involved in transcription. Neutrophils treated for 4h with media alone orR-roscovitine (20μM) were subjected to RNA isolation using Nucleospin kit and Trizol extraction prior to interrogation by microarray. Bar graph above shows genes down-regulated >2 fold by R-roscovitine compared to control (-), genes up-regulated >2 fold by R-roscovitine treatment compared to control (+) and genes unaffected. Heat map shows gene expression most changed (upregulated and downregulated) across the four treatment groups (R-roscovitine (R), R-roscovitine/LPS (RL), Control(C) and LPS alone (L)).Blue colour denotes downregulation and Yellow is upregulation.

Figure 5.13: Effect of CDK inhibitor drugs on neutrophil transcriptome

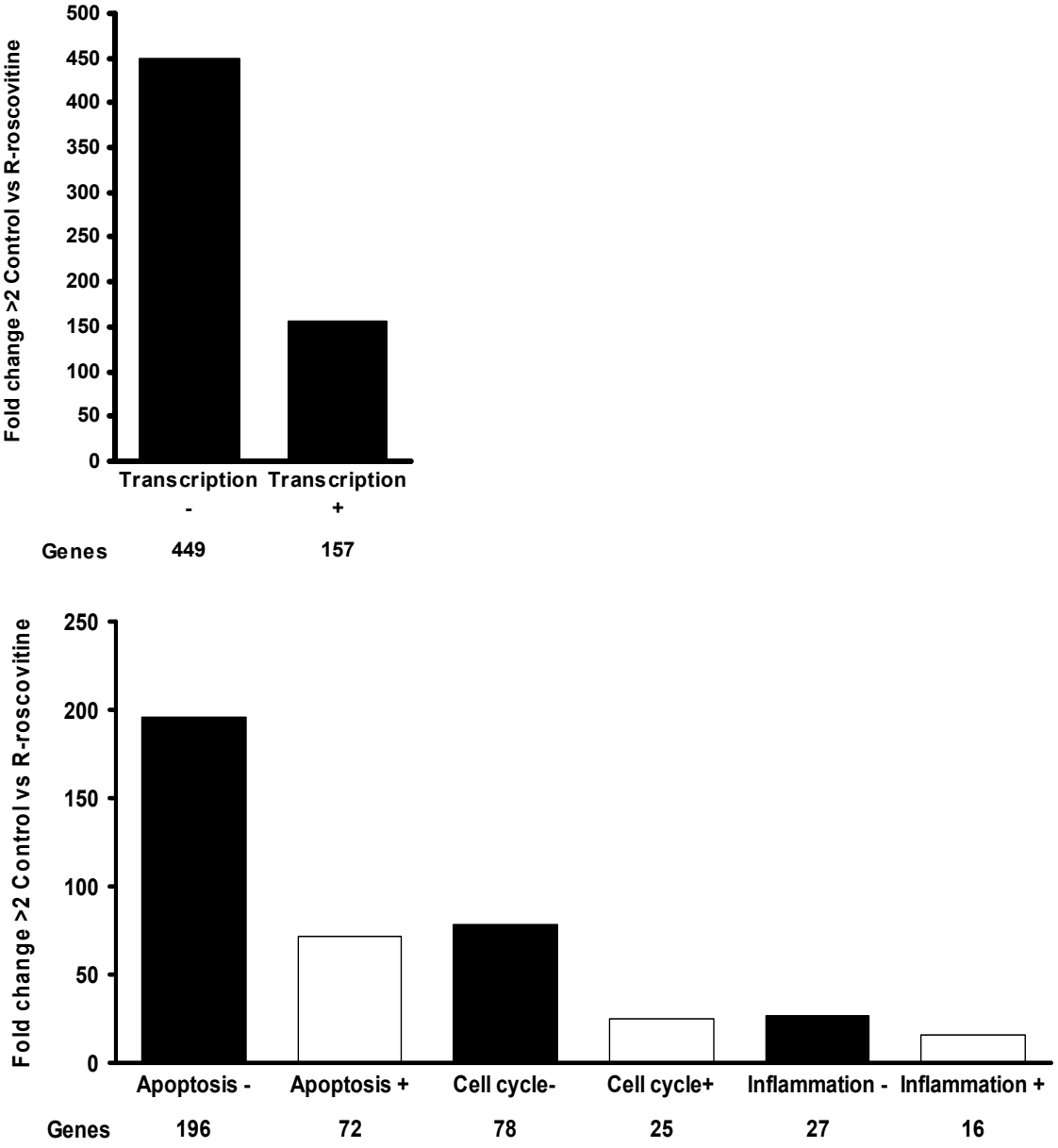


Figure 5.13: Illumina gene chip effect of CDK inhibitor drugs on specific clusters of genes relevant to this thesis. Neutrophils treated for 4h with media alone or R-roscovitine (20μM) were subjected to RNA isolation using Nucleospin kit and Trizol extraction prior to interrogation by microarray. Results show genes down-regulated >2 fold by R-roscovitine treatment compared to control (-) and genes up-regulated >2 fold by R-roscovitine compared to control(+). Gene families assessed include: transcription, apoptosis, cell-cycle and inflammation.

Figure 5.14: Gene ontology (GO) terms over-represented in genes up/down-regulated following neutrophil treatment with R-roscovitine

	Downregulated genes (>2 fold p<0.01)	Upregulated genes (>2 fold p<0.01)
1	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	translational elongation
2	cellular metabolic process	cellular protein metabolic process
3	cellular catabolic process	ribosomal small subunit biogenesis
4	**positive regulation of I-kappaB kinase/NF-kappaB cascade**	attachment of GPI anchor to protein
5	response to unfolded protein	**immune response**
6	protein transport	rRNA processing
7	**regulation of programmed cell death**	generation of precursor metabolites and energy
8	regulation of primary metabolic process	**leukocyte activation during immune response**
9	protein modification by small protein conjugation or removal	lysosomal transport
10	modification-dependent protein catabolic process	**negative regulation of interleukin-8 biosynthetic process**
11	regulation of macromolecule metabolic process	**positive regulation of pinocytosis**
12	**regulation of transcription from RNA polymerase II promoter**	**defense response to fungus**
13	positive regulation of nitrogen compound metabolic process	**leukocyte activation**
14	positive regulation of biosynthetic process	regulation of translation
15	protein localization	phospholipid biosynthetic process
16	positive regulation of biological process	**antigen processing and presentation**
17	**cell cycle**	negative regulation of peptidase activity
18	RNA splicing	**programmed cell death**
19	small GTPase mediated signal transduction	glycerolipid biosynthetic process
20	**mRNA processing**	biosynthetic process
21	cellular protein localization	**lymphocyte differentiation**
22	**transcription initiation from RNA polymerase II promoter**	**T cell activation**
23	**positive regulation of transcription**	**NADPH regeneration**
24	**induction of apoptosis via death domain receptors**	**regulation of macrophage activation**
25	**RNA biosynthetic process**	cellular macromolecule biosynthetic process

Figure 5.14: Over-represented gene ontology terms in genes significantly up and down-regulated (up and down-regulation >2-fold, R-roscovitine compared to control with significance of p<0.001) R-roscovitine:Control.

5.6 Discussion

The discovery that neutrophils could be driven to undergo apoptosis by pharmacological inhibitors of elements of the cell-cycle machinery termed cyclin-dependent kinases (CDKs) has established a major research track for our group (Duffin et al. 2009;Leitch et al. 2009;Leitch et al. 2010b;Rossi et al. 2006). This finding was unexpected given the cell-cycle status of neutrophils as the only other terminally differentiated cell type (neurons) studied with these pharmacological agents, prior to our work, demonstrated enhanced longevity (Maas, Jr. et al. 1998). Additionally it had been shown that some CDKs were progressively down-regulated as myeloid lineage cells matured (Klausen et al. 2004). Myeloid lineage cells differentiate in the following order: myeoblast, promyelocyte, myelocyte, metamyelocyte, band cell, segmented cell, neutrophil. Klausen et al., using primary human bone-marrow cells from healthy volunteers described progressive loss of CDKs 2, 4 and 6 which began at the myelocyte/metamyelocyte stage and was almost complete by full maturation (Klausen et al. 2004). This occurrence coincided with upregulation of the endogenous CDK inhibitor p27kip1 and both results were shown at the mRNA and protein level. No consistent finding with regard to the up or down-regulation of the CDK binding partners, termed cyclins, was obtainable illustrating the complexity of regulation involved in this system. This finding was important as other works on granulopoiesis had utilised a variety of cell-lines and discovered, perhaps unsurprisingly, a variety of CDK/CDK binding partner expression patterns. It also suggested that p27kip1 might be more important than the commonly implicated endogenous CDK inhibitor, p21. Interestingly, p21 has come to the fore recently as a novel modulator of the response to LPS made by inflammatory macrophages (Lloberas et al. 2009;Scatizzi et al. 2009).

The previous chapters have characterised the molecular mechanism by which CDK inhibitor drugs drive neutrophil apoptosis (Chapter 3) and identified functional CDKs and transcriptional machinery in neutrophils (Current Chapter). In addition they have identified specific transcriptional effects of CDK inhibitor drugs on neutrophils. The

evidence gathered so far has shown that apoptosis proceeds via the intrinsic pathway, that short-lived, responsive mRNAs are selectively affected by CDK inhibitor drugs and that proteins involved in apoptosis are amongst that cluster of transcripts.

I have identified at gene and protein level the expression of CDKs, their binding partners and endogenous inhibitors in neutrophils. Perhaps unsurprisingly given the terminally differentiated state of these cells the most significantly expressed CDKs, CDKs 7 and 9, have no role in the cell cycle/proliferation but are essential for transcription of a key subset of genes (Roy et al. 1994;Zhu et al. 1997). Gene expression is the end-product of a number of different processes (transcription, mRNA elongation/splicing/capping, translation, degradation) but transcription regulated by a variety of transcription factors is often the most important contributor. The standard model of transcription from a promoter involves the binding and formation of a pre-initiation complex composed of RNA pol II and various TATA box binding proteins, kinases and ubiquitin ligases. This is termed the RNA pol II holoenzyme and is responsible for the majority of RNA transcription in mammalian cells. The pre-initiation complex requires further input before full-length, viable mRNA can be produced. It is at this stage that regulation from signalling cascades such as those activated in the presence of inflammatory stimuli is integrated into the system. Signalling cascades may promote the subcellular redistribution of transcription factors (e.g. NF-κB) or may activate kinase cascades by dimerisation or ligand-binding. It is the subsequent binding and activation of the pre-initiation complex by pathway-specific transcription factors and coactivators as well as phosphorylation by dedicated kinases that determines the level and variety of gene expression. In highly inducible genes such as those involved in inflammatory signalling or apoptosis RNA polymerase II recruitment and activation occur simultaneously. These genes appear to be heavily dependent on the positive transcription elongation factor b (P-TEFb) complex. P-TEFb is a heterodimer of CDK9 and cyclin T1 that can phosphorylate serines 2 and 5 within the 52 repeats of heptapeptide YSPTSPS on the CTD of RNA pol II. Interestingly, it has been shown that NF-κB dependent genes are dependent on P-TEFb activity and that there may be a physical interaction between NF-κB and P-TEFb (Barboric et al. 2001;Nowak et al. 2008). Phosphorylation of serine 2 is

known to be important for transcriptional elongation of highly inducible genes. CDK7, cyclin H and the cofactor MAT1 are part of the transcription factor IIH complex (TFIIH) which is known to be important for the initiation of transcription. Interestingly, it appears that LPS stimulation enhances transcription of the CDK7 gene suggesting that enhanced transcription directly drives production of the machinery of transcription (figure 5.4). This process is down-regulated by the CDK inhibitor drug, R-roscovitine. Additionally LPS stimulation appears to enhance CDK9 and RNApol II recruitment to decondensed/transcriptionally active chromatin within the nucleus, a process that is unaffected by CDK inhibitor drug treatment.

It is my contention that it is inducible genes: short-lived, rapidly upregulated, responsive and dependent on CDK activation that confer the phenotype that allows neutrophils to be an effective early arm of immune defence. The gene clusters that fit this description include those involved in transcription, apoptosis, signalling and chemokine production (figure 5.10). To support this contention I have demonstrated that RNA polymerase II is both present in neutrophils and phosphorylated by CDKs 7 and 9 in order to enhance transcriptional capacity (figure 5.7). Inhibition of CDKs 7 and 9 leads to dephosphorylation of this enzyme and downregulation of transcription. As I have previously discussed the transcriptional capacity of neutrophils has been a matter of some controversy. Work by Cassatella and MacDonald et al (Cassatella 1995;McDonald et al. 1998) suggested that neutrophils do indeed have the capacity for *de novo* transcription. Here I describe the machinery by which they achieve this functionality and a method by which it may be selectively altered to enhance apoptosis and resolution of inflammation.

Summary of Chapter 5

1. **CDKs 2,5,7,9 present in human neutrophils** *as assessed by western blotting, illumina gene chip technology and confocal microscopy*
2. **CDKs intracellular localisation can be dictated by known neutrophil survival factors**
3. **RNA polymerase II is present and functional in human neutrophils** *as assessed by western blotting and confocal microscopy*
4. **CDK inhibitors can prevent phosphorylation of RNA polymerase II by CDKs 7 and 9** *as assessed by western blotting*
5. **Gene expression in human neutrophils is specifically regulated by CDK inhibitor drug treatment** *as assessed by illumina gene chip technology*

6. Results: A molecular biology strategy to corroborate data generated with regard to pharmacological CDK inhibitor drugs

In order to strengthen our hypothesis that CDKs 7 and 9 are of key importance in the promotion of neutrophil apoptosis by CDK inhibitor drugs we aimed to develop a molecular biology knockdown strategy that would be successful in human neutrophils.

Human neutrophils are notoriously difficult to influence by techniques such as antisense, siRNA and viral vector transfection that have become established standards in other cell-types. This is because neutrophils have a constitutive life-span of approximately 24h that can be extended in response to infection/inflammation and certain pharmacological agents (dexamethasone, db-cAMP). This doesn't leave a satisfactory window for achieving cell transfection and gene of interest knockdown. However, antisense has been used to knockdown the key survival protein Mcl-1 in neutrophils subject to enhanced longevity because of dexamethasone (Sivertson et al. 2007) treatment or hypoxia (Leuenroth et al. 2000b) and recently Sabroe et al have demonstrated that a lentiviral strategy can be used successfully to knockdown the NF-κB pathway (Dick et al. 2009). Certain publications have described the use of siRNA in neutrophils but these data are controversial in my opinion (Azuma et al. 2007;Ernens et al. 2006). In the first publication phospholipase-D is knocked down in rat neutrophils over a 24h period before cells are stimulated with fMLP (Azuma et al. 2007). In my experience the interpretation of these data would be rendered difficult by a high degree of cell death, both apoptotic and necrotic at this time-point. The second paper describes gene knockdown in human neutrophils over a 48h period prior to stimulation with adenosine and fMLP (Ernens et al. 2006) which requires an *in vitro* neutrophil life-span not compatible with the majority of literature dedscribing unstimulated neutrophils. It may be the case that the siRNA process significantly extends neutrophil lifespan through off-target effects (e.g., described effects on the JAK/STAT pathway (Sledz et al. 2004)) but

in that case the experiment requires significantly better design and control to justify its use let alone conclusions.

The most consistently employed knockdown strategy in neutrophils (and eosinophils) is the HIV-tat transduction method (Gump et al. 2007). This bypasses the necessity for knockdown at the intracellular gene transcription level by delivering a pre-synthesised protein direct to the cell. The tat protein is an HIV virulence factor that promotes cell-membrane entry probably by a novel process (Gump et al. 2007). Small peptides to large proteins can be coupled to a short peptide sequence of the tat protein and efficiently transduced into a variety of cell types.

I chose to use the HIV-tat transduction system to deliver dominant negative CDK 7 and 9 proteins to human neutrophils in order to test our hypothesis that CDK7 and 9 were important targets of CDK inhibitor drugs in neutrophil apoptosis and the resolution of inflammation.

6.1 Cloning of HIV-tat dominant negative CDKs 7 and 9

In order to generate HIV-tat dominant negative (dn) CDKs 7 and 9 protein it was necessary to generate a DNA sequence that combined both HIV-tat, dnCDK 7 or 9 and a His-tag sequence to allow affinity purification of the protein later in the process. HIV-tat plasmids (plasmid sequence in figure 6.1) were a generous gift from Dr Steven Dowdy (UCSD) and CDK7dn and 9dn (plasmid maps in figure 6.2) were bought (for a nominal fee) from Addgene with the permission of the Fujinaga lab. The CDKdn DNA was sub-cloned into the HIV-tat plasmid as described on page 82 (methods section). The success of this process was confirmed by restriction enzyme digest which released an appropriately sized fragment (figure 6.3i), PCR for relevant CDKdn (figure 6.3ii) and finally by definitive sequencing using 5 sets of primers (named CDK FOR 1 and 2, CDK REV 1 and 2 and T7- see materials for sequences). Figure 6.4 shows key sections of the obtained sequence which fully aligned with known sequences for the tat plasmid and the

CDKdn. In addition it was possible to confirm that the one amino acid substitution conferring dominant negative properties was present as required (figure 6.4i).

Figure 6.1: pET 28b Tat 2.1 plasmid

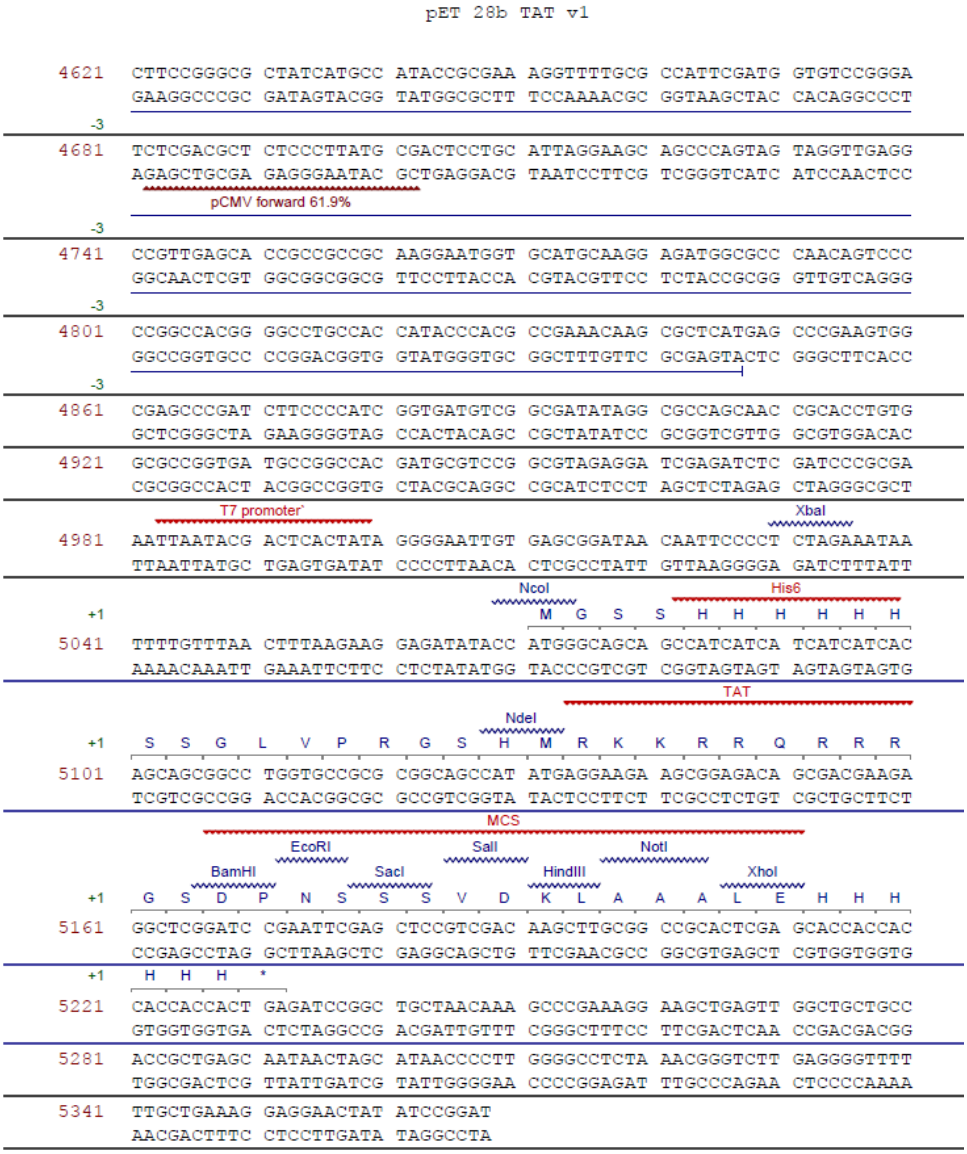
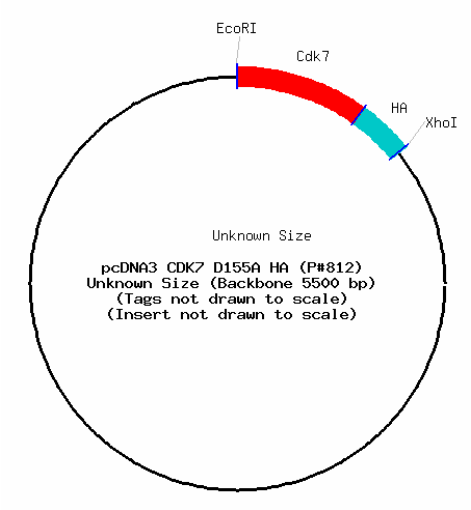


Figure 6.1: Plasmid sequence for pET-tat 2.1 provided by Dr Steve Dowdy (UCSD).

Figure 6.2: pLINK CDK7-DN and pLINK CDK9-DN plasmids



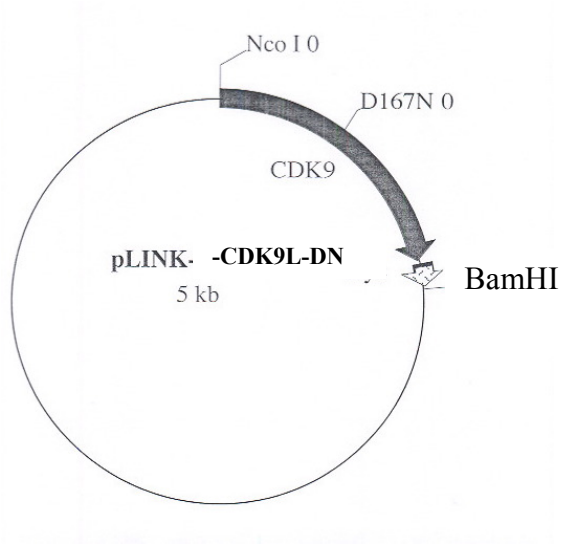
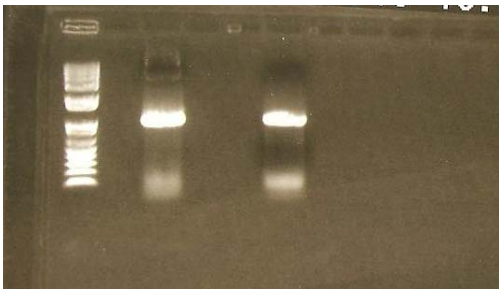


Figure 6.2: Vector maps for pLINK-CDK7 and 9 DN kindly provided by Koh Fujinaga via Addgene plasmid services.

Figure 6.3: Confirmation of correct cloning of CDK9-dn to Tat plasmid

i)

Lane 1 2 3



ii)

Lane 1 2 3 4

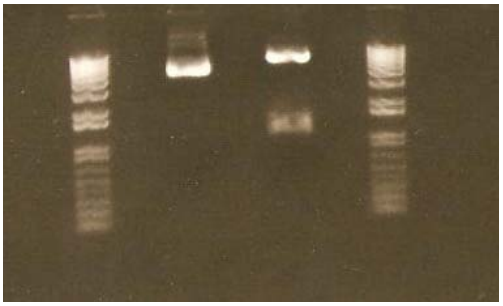
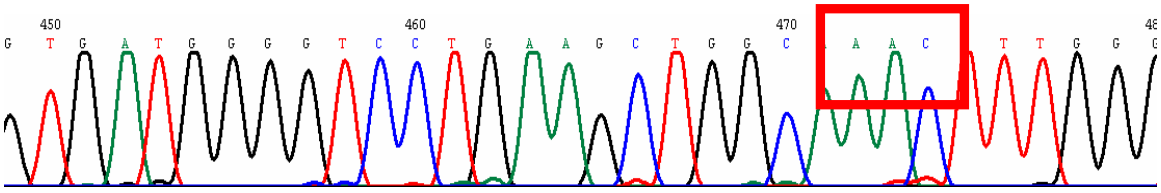


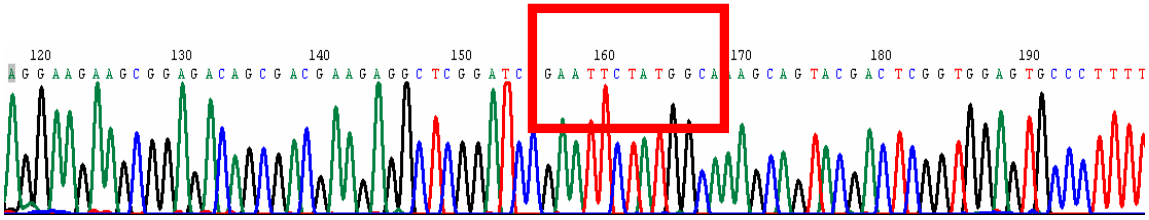
Figure 6.3: In i) Cloning of CDK9-dn to tat plasmid is confirmed by PCR using primers FOR1 and REV1 (see materials page 74) Lane 1 is marker, lane 2 is TopoXL vector with CDK9dn and lane 3 is tat vector CDK9dn . In ii) a restriction enzyme digest using EcoRI/NotI was performed to release the CDK9dn fragment which had been cloned in with the use of these restriction sites. In lane 1 is marker, lane 2 is uncut tat plasmid, lane 3 is plasmid cut with restriction enzymes and lane 4 is marker.

Figure 6.4: Sequencing of tat-CDK9dn plasmid

i) Inactivating enzymatic domain of CDK9: GAC-AAC(D-N) = Aspartic acid to asparagine



ii) Clone 6: Tat vector-EcoRI-CDK9-DN start



iii) Clone 6: CDK9-DN-NotI-His6tag

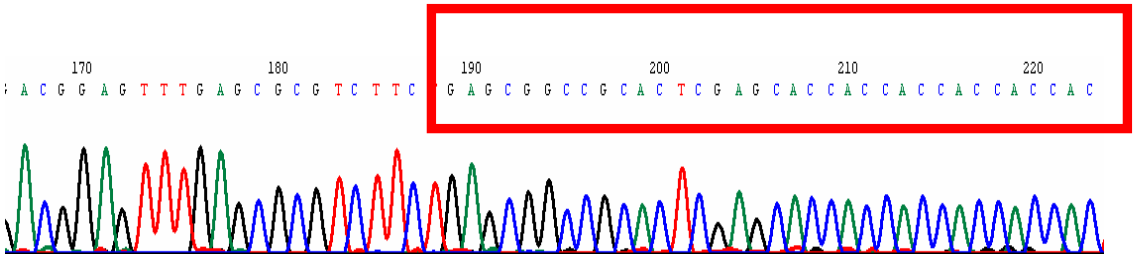


Figure 6.4: Excerpts from sequencing results for Tat-CDK9-DN DNA. Full sequencing was performed for both Tat-CDK-DN DNA and representative images for CDK9-DN are shown. In i) the dominant negative amino acid substitution is identified, in ii) tat vector followed by the EcoRI restriction enzyme sequence and the start of CDK9-DN are identified and finally in iii) the His-6 tag is confirmed present.

6.2 Production of HIV-tat dominant negative CDK7 and 9 proteins

HIV-tat proteins were produced by transformation of BL21(DE3)plysS *E.coli* bacteria. A variety of conditions were investigated to establish whether it might be possible to produce soluble protein without the use of denaturing conditions. *E.coli* were induced to produce protein at temperatures of 20^{o}C, 30^{o}C, 37^{o}C over time periods from 2-24 hrs and in the presence/absence of glucose. All experiments were carried out under the selection pressure of kanamycin (HIV-tat-dnCDK7/9 confer resistance). It appeared that regardless of the above conditions the majority of protein was contained in insoluble inclusion bodies (as has previously been found by Zhao et al). It was determined that in order to produce enough protein to allow for losses incurred in purification steps denaturing conditions would have to be used. CDK7 and 9 tat proteins were solubilised and then subjected to His-tag affinity purification using batch metal affinity chromatography (Talon^{TM} Cobalt resin) as shown in coomassie stained gel in figure 6.5.

During batch isolation *E.coli* lysates were solubilised in 8M urea or 6M Guanidium HCl and bound to cobalt metal affinity resin using conditions described in method. Proteins were eluted with the use of 150mM to 1M imidazole and checked by coomassie staining and western blotting for both CDK7/9 and His-tag. Figure 6.6 shows representative images of western immunoblotting for His-tag and CDK9 (for example).

It was confirmed that dn-CDK7 and 9 tat proteins had been produced. However subsequent HPLC purification and salt exchange steps demonstrated that the majority of tat protein produced aggregated or degraded on introduction of non-denaturing buffer. This was despite the use of various strategies employed to produce pure protein in cell compatible buffer including: spin column dialysis, overnight dialysis by slide-a-lyzer/snakeskin tubing, HPLC on column refolding (various buffers and strategies), HPLC gel filtration and ion exchange chromatography.

Figure 6.5: Coomassie gel showing tat-CDK9dn protein batch isolation process

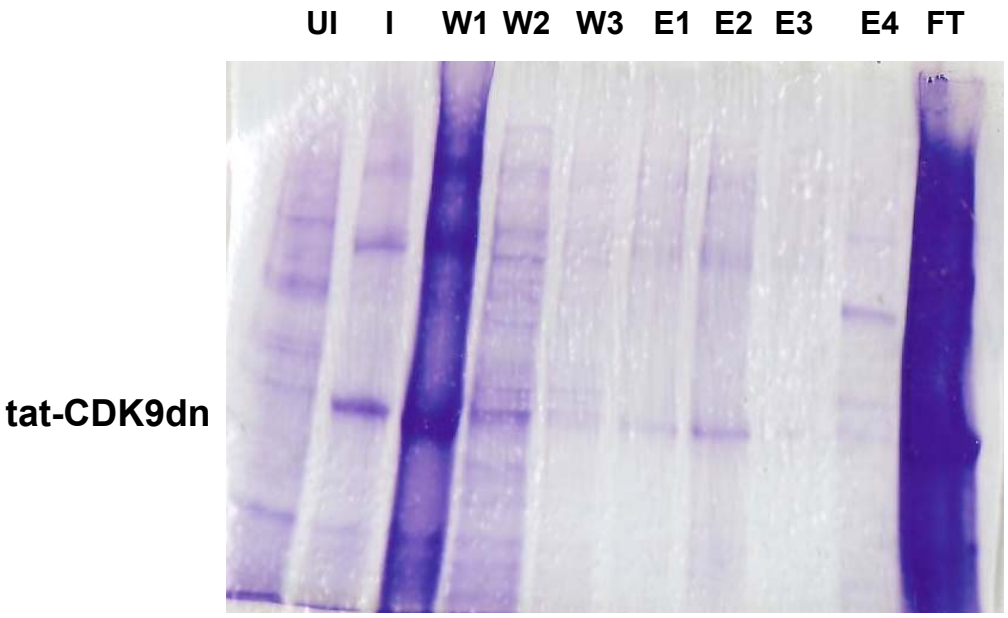


Figure 6.5: Coomassie protein stain of gel showing batch isolation process. Protein was produced by BL21 E.Coli induced (I) with the use of IPTG (1mM). Uninduced (UI) BL21 E.coli protein lysate is shown for comparison. Protein was bound to cobalt resin using a batch method and the resin was subsequently washed 3x (W1-3) in wash buffer prior to elution (E) with imidazole (150mM) buffer. Elution was repeated 4 times (E1-4). FT represents flow through following binding of initial protein sample to resin.

Figure 6.6: Western blot for CDK9 and His-tag

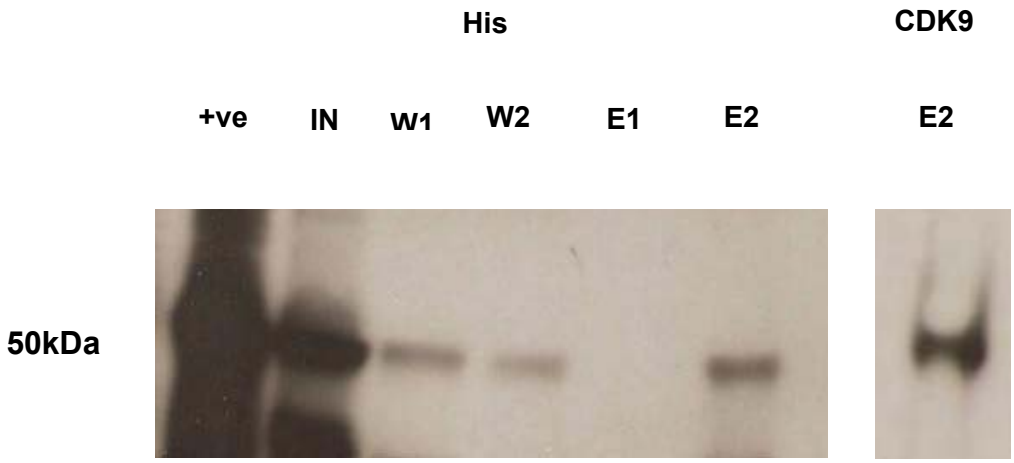


Figure 6.6: Western blots for His-tag (His) and CDK9. Loaded samples are the same as those shown in figure 6.5 (Coomassie stain). Positive control for CDK9 is A549 lysate. Protein was bound to cobalt resin using a batch method and the resin was subsequently washed 3x in wash buffer, washes 1 and 2 (W1+2) are shown. Elution (E) samples E1 and E2 from previous figure are probed for His-tag and E2 is subsequently probed for CDK9.

6.3 Isolation and purification of HIV-tat dominant negative CDK7 and 9

HPLC affinity purification using nickel and cobalt IMAC columns was attempted but was unsuccessful. For reasons that I have been unable to determine His-tag binding was not of strong enough affinity to allow the wash steps required for HPLC purification. The chromatograph shown in figure 6.7 shows no protein peak as determined by UV trace. The peak in the latter stages represents the change in trace related to imidazole content of elution buffer (Absence of protein of interest was confirmed by agarose gel electrophoresis of eluted samples followed by coomassie staining). Coomassie staining of agarose gel electrophoresis of samples from various stages of process demonstrated that the majority of protein was lost during the wash stage. This seemed to occur with cobalt and nickel metal ion columns, independently of buffer conditions (guanidium, urea or NDSB-201, pH, imidazole content, salt content) and time of contact of protein sample with resin. In order to proceed without returning to DNA level I attempted to batch purify protein using metal affinity followed by clean-up using HPLC gel filtration and ion exchange. Although adequate sample was obtained by batch purification, clean-up stages were complicated by significant loss of protein to aggregation and insolubility. Figures 6.7 and 6.8 show chromatograms of attempted clean-up steps. The protein of interest should have appeared between 50 and 60ml (as compared with known protein standards) by gel filtration but appeared earlier in aggregate forms as detected by UV trace and agarose gel electrophoresis. In this experiment the protein came out of solution when it was filtered directly into non-denaturing buffer but in a repeat using a gradient of denaturing to non-denaturing buffer the protein also came out of solution (Gu et al. 2001;Gu et al. 2002).

Figure 6.7: High performance liquid chromatography clean-up of tat-CDK9dn

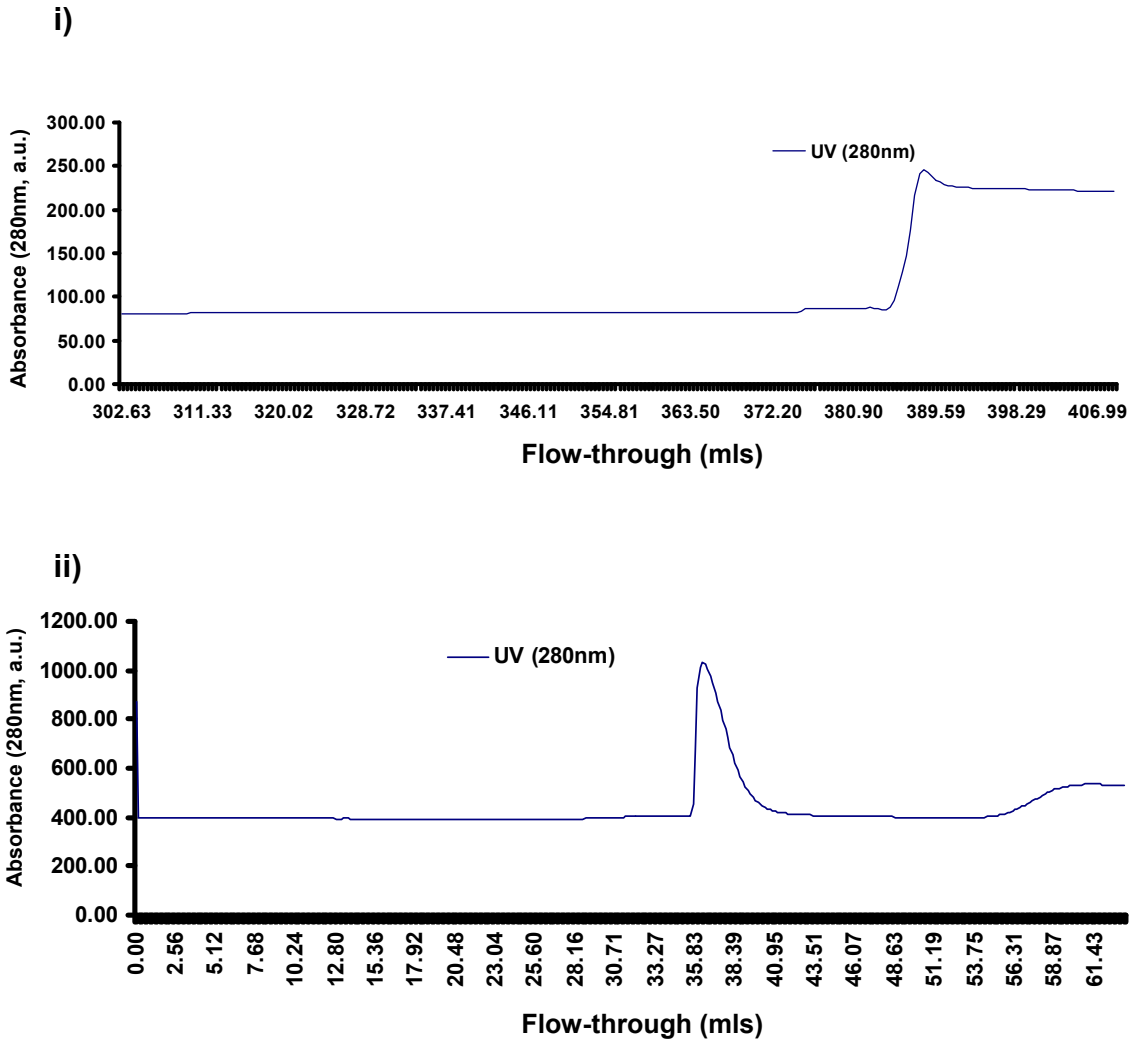


Figure 6.7: UV traces from HPLC tat-CDK9dn protein isolated from E.coli lysates. In i) protein was bound to a Cobalt IMAC column according to method 1 (see materials and methods). Trace shows no purified protein peak. In ii) protein isolated from E.coli lysates was subjected to HPLC gel filtration through a buffer gradient as per method 2. The protein peak should appear between 50 and 60mls (by comparison with known standards but instead appears earlier (35-40mls) suggesting protein aggregation.

Figure 6.8: HPLC purification of tat-CDK9dn by gel filtration and ion exchange chromatography

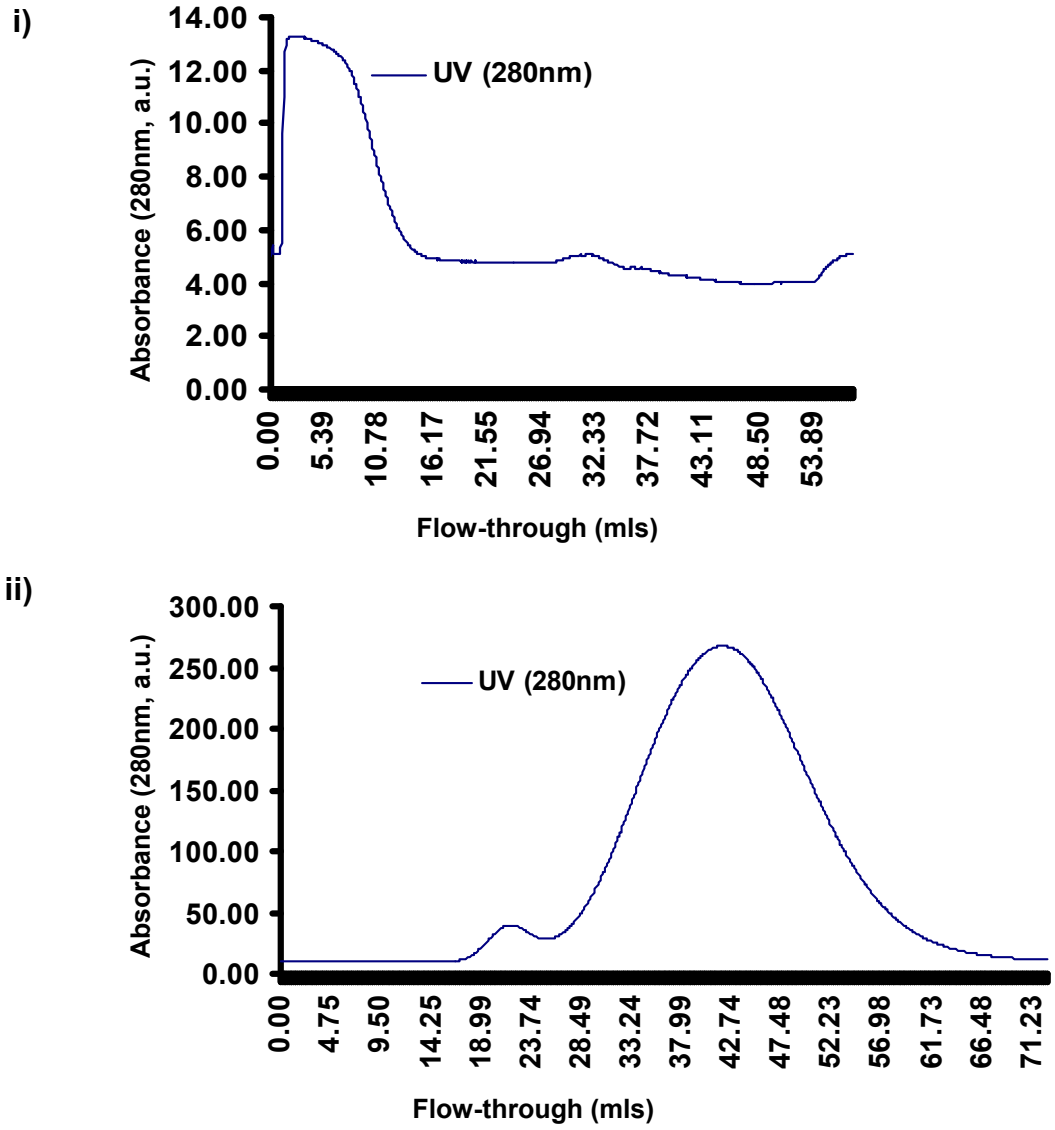


Figure 6.8: UV traces from HPLC tat-CDK9dn protein isolated from E.coli lysates. In i) protein isolated from E.coli lysates was subjected to HPLC gel filtration into refolding buffer as per method 3. The protein peak should appear between 50 and 60mls (by comparison with known standards but instead appears earlier (0-10mls) suggesting protein aggregation. In ii) protein purification by ion exchange chromatography was attempted.

6.4 Transduction of neutrophils by HIV-tat dominant negative CDK7 and 9

I decided to test the ability of the small amounts of tat protein produced in soluble form to transduce into human neutrophils as a proof of concept experiment. A quantity of protein was labelled with FITC which allowed assessment of cellular location by confocal microscopy and flow cytometry. In parallel assays for neutrophil apoptosis were performed with unlabelled protein. It was clear that FITC labelled protein could transduce human neutrophils figure 6.9 and 6.10. It was also demonstrated that proteins significantly activated human neutrophils (see figure 6.9 and compare morphology of cells between free FITC treated and FITC labelled tat-CDK9dn) which made interpretation of flow cytometry assay for neutrophil viability difficult (data not shown). It was subsequently established by limulus assay that this was related to LPS contamination of the protein associated with the batch method of protein purification. It was determined that further purification of tat proteins by HPLC is required before they can be successfully used in human neutrophils. An endotoxin affinity column was used to promote removal of this important bacterial product. The protein of interest bound strongly to the endotoxin column along with LPS and could not be eluted in sufficient quantity to allow successful buffer exchange.

Figure 6.9: HIV-tat transduction of human neutrophils using inadequately purified, FITC labelled tat-CDK9dn protein

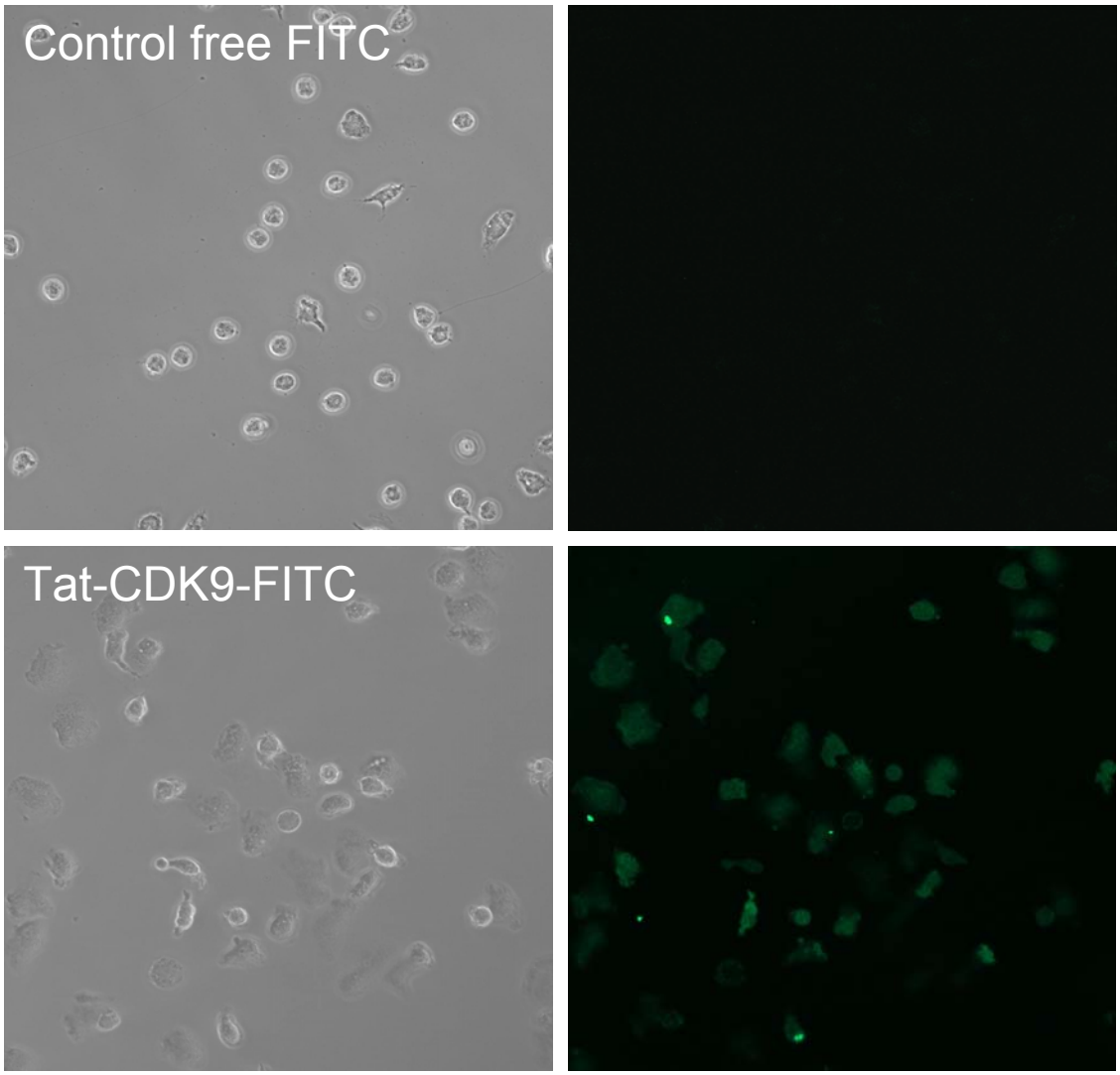


Figure 6.9: tat-CDK9dn protein was labelled with FITC and labelled protein was purified by buffer elution from PD-10 gel filtration column. Human neutrophils isolated as previously described were exposed to FITC-labelled tat-CDK9dn protein or free FITC for 30min in a 2ml eppendorf at 37°C, 5% CO_2. Cells were subsequently spun down and washedx3 in medium + 10% autologous serum. After washing cells were transferred to coverslips and prepared for confocal microscopy (Zeiss LSM meta 510, 488nm laser, x400 oil immersion).

Figure 6.10: HIV-tat transduction of human neutrophils using inadequately purified, FITC labelled tat-CDK9dn protein

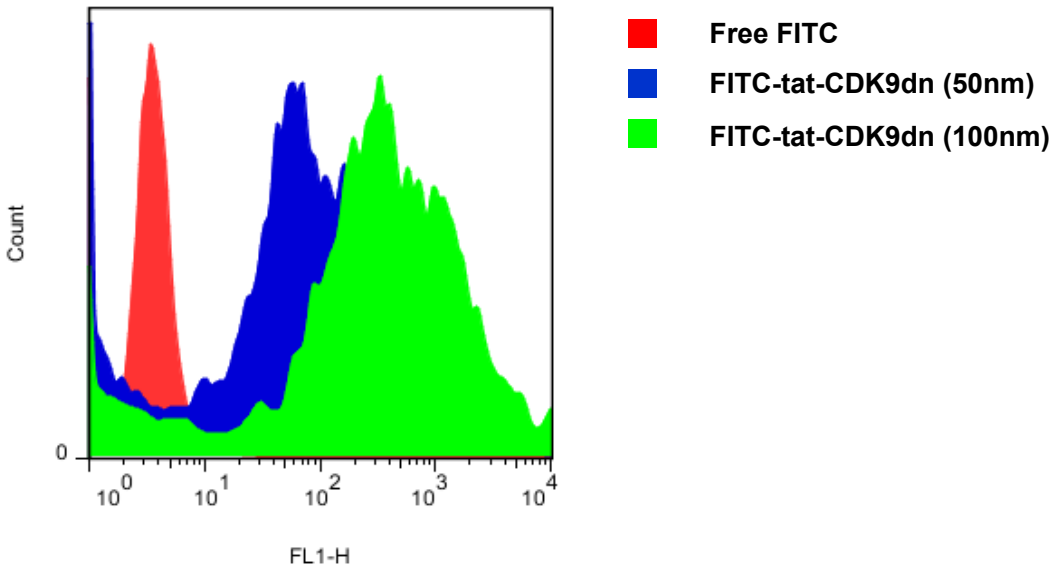


Figure 6.10: tat-CDK9dn protein was labelled with FITC and labelled protein was purified by buffer elution from PD-10 gel filtration column. Human neutrophils isolated as previously described were exposed to FITC-labelled tat-CDK9dn protein or free FITC for 30min in a 2ml eppendorf at 37°C, 5% CO_2. Cells were subsequently spun down and washedx3 in medium + 10% autologous serum. After washing cells were analysed by confocal microscopy for shift in FL-1 indicating incorporation of FITC dye. Two concentrations of labelled tat-CDK9dn protein are shown (50nm and 100nm) and compared to free FITC (which should not be internalised).

6.5 Discussion of Chapter 6

The HIV-tat transduction system has been successfully used to deliver peptides and large proteins to both neutrophil and eosinophil granulocytes (Fujihara et al. 2002;Han et al. 2003;Kilpatrick et al. 2010a;Kilpatrick et al. 2010b;Vidarsson et al. 2006). The advantage of this method is that it delivers a pre-formed peptide/protein to the cell without the extra steps of gene knockdown required by techniques like lentiviral transfection, antisense knockdown and siRNA. Theoretically it should also be possible to deliver the protein of choice without recourse to transfection agents that might have negative effects on cell longevity (there is a degree of cell-toxicity associated with most transfection protocols). This is important when a major read-out of the experiment is the promotion of cell death. The numerous examples of successful transduction of granulocytes and the theoretical advantages detailed above led me to pursue an HIV-tat transduction strategy for the knockdown of CDK 7 and 9 function in human neutrophils.

Dominant negative CDKs 7 and 9 are described in the literature and have been used to successfully knockdown the functions of these proteins including there effects on transcription (Garriga et al. 1998;Garriga et al. 2004;Garriga et al. 2009;Zhu et al. 1997). The experimental plan was to produce tat-linked CDKdn and then to transduce neutrophil granulocytes and repeat the experiments already described using pharmacological CDK inhibitors. In short, we planned to perform assessments of neutrophil viability over a time-course following transduction (flow cytometry for annexin-V/PI and morphological assessment by cytocentrifuge preparation), to measure RNApol II phosphorylation (western blotting) and Mcl-1/Bim levels (PCR and western blotting). An initial preparatory experiment would have been to FITC label tat-CDKdn and ensure it had entered cells by flow cytometry assessment and fluorescence microscopy. In addition we planned to perform western blots for His tag and CDK (it might be expected that native and recombinant protein would have different molecular weight because of the His and tat tags) in transduced cells to confirm protein internalisation.

The decision to attempt IMAC purification of tat-CDKdn protein was made because this technique had previously been utilised by other groups (Zhang et al. 2009;Zhao et al. 2007) and because the tat-plasmid has a His-tag sequence built in (see figure 6.1). For reasons we haven't been able to identify but presumably related to the properties of CDK proteins this method was only partially successful. The affinity of the His-tag for the IMAC column appeared to be significantly decreased and only batch purification by metal ion resin proved possible and even by this method there was still significant protein loss in the washing stages. Batch-purified protein was found to be significantly contaminated by LPS (proven by LAL assay and see figure 6.7) but was capable of transducing human neutrophil granulocytes (as proven by FITC labelling, confocal microscopy and flow cytometry).

HPLC purification of the protein was attempted by gel filtration (size exclusion) and ion exchange chromatography. Despite using various well-described buffers and methods (see materials and methods) refolded protein in monomeric form wasn't obtained in sufficient quantities for experimental use.

This experimental line has not been exhaustively investigated however and there are still numerous approaches that could be used to overcome the problems encountered. It has been noted that different proteins require specifically tailored purification strategies though the means to achieve this consistently without resorting to trial and error has not been developed (Buckle et al. 2005). It has been estimated that only 13% of human proteins are isolated in soluble form from genetically modified bacteria (Braun et al. 2002). There are a number of variations on the gel filtration/ion exchange strategies that I have not been able to attempt that might potentially allow good yield and acceptable purity. In addition, the use of an ATP-binding column is a strategy often used in the isolation of kinase-type proteins and this strategy has been used to successfully isolated CDK proteins previously. Alternatively, it would be possible to start again with a different expression vector in order to avoid some of the contamination issues associated

with bacterial expression. Other potential expression strategies might include baculovirus, mammalian, plant or yeast expression (Demain et al. 2005). Given the inefficiency of the his-tag the introduction of a different tag isolation method such as GST, CBP, CYD (covalent yet dissociable NorpD peptide), Strep II, FLAG or HPC might be contemplated (Lichty et al. 2005).

Additionally, it might be possible to subject stimulated (and therefore longer-lived) neutrophils to lentiviral knockdown in a carefully controlled experiment akin to that recently achieved by Sabroe et al (Dick et al. 2009).

6.6 Summary of Chapter 6

1. **Cell-delivery peptides coupled to dominant negative CDKs 7 and 9 have been produced but require further purification**
2. **Future approaches for the production of highly purified proteins may involve alternative protein production/isolation techniques**
3. **Lentiviral/siRNA transduction of stimulated neutrophils may merit consideration**

Chapter 7: CDK inhibitor drugs and the bleomycin lung injury model

7.1 CDK7/9-specific inhibitor DRB drives resolution of inflammation in the bleomycin lung injury model

In order to enhance our previous work with the CDK inhibitor, R-roscovitine in the resolution of neutrophil-dominant lung injury (Rossi et al. 2006) we attempted to refine the specificity of the pharmacological agent used. There are a number of different classes of CDK inhibitor drug that show varying specificities for different CDKs. At high concentrations these specificities disappear but by careful use within designated concentration ranges it ought to be possible to target defined CDKs. The CDK inhibitor drug DRB has been described as a CDK7/9 specific inhibitor and its utility in this regard has been described in numerous publications in the literature (Lee et al. 2001;Luecke et al. 2005;Marshall et al. 1996;Peng et al. 1998). It has also been used as an inhibitor of transcription, a function it has been shown to perform by the inhibition of CDKs 7 and 9 (Lee et al. 2001;Luecke et al. 2005;Marshall et al. 1996;Peng et al. 1998). In this thesis DRB has been shown to drive human neutrophil apoptosis with equal efficacy and similar pharmacokinetics to R-roscovitine (figure 3.6, page 101) and to activate caspases (figure 3.10, page 108). In addition, we have shown that DRB drives apoptosis of mouse bone-marrow derived neutrophils *in vitro* (data not shown).

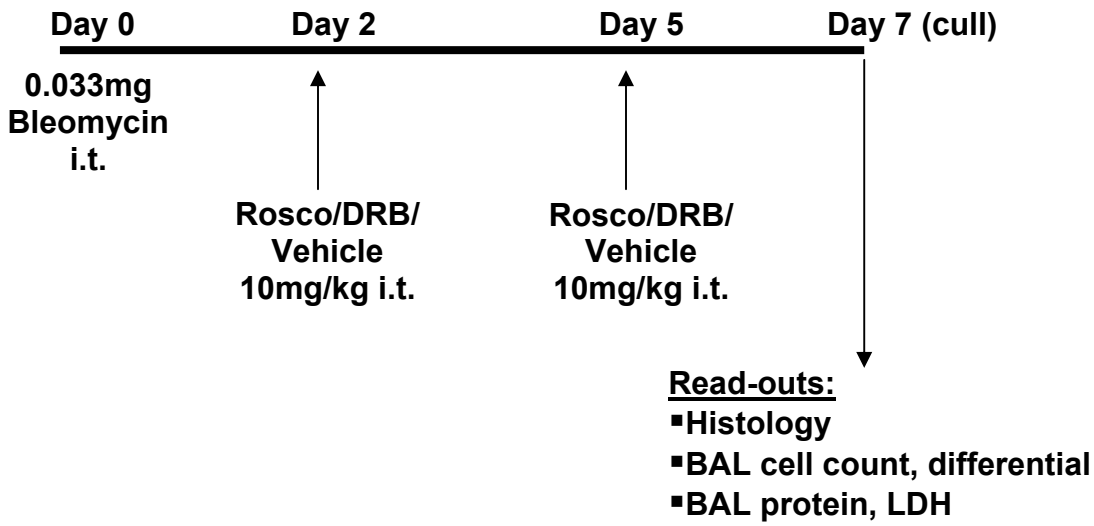
The model of neutrophil-dominant lung injury we chose to investigate was the acute-phase of bleomycin-induced lung injury. This model has been the gold-standard for investigation of the lung inflammation and fibrosis associated with IPF and has been shown to be reproducible and similar to human disease pathology (It causes acute alveolar/interstitial inflammation with subsequent development of epithelial cell death and basement membrane damage leading to predominantly bronchiolocentric fibrotic changes (Moeller et al. 2008;Moore et al. 2008)). The initial phase of the injury up to

Day 7 resembles the histopathology of acute exacerbation of IPF or even ARDS/ALI and it is therefore a useful model for the investigation of neutrophil-dominant inflammation.

I investigated the ability of DRB to resolve neutrophilic inflammation associated with the bleomycin lung injury model. This seemed a logical approach because of our previous research finding that R-roscovitine could promote resolution of the neutrophil-dominant inflammation associated with this model. In that experiment mice were given *i.t.* bleomycin on Day 0, R-roscovitine was administered *i.p.* at a dose of 100mg/kg on day 2 and mice were killed on day 7. Histological evidence of neutrophil-dominant inflammation was clear in control animals but in R-roscovitine-treated animals, inflammation had almost entirely resolved.

To increase the novelty of this essentially mechanistic experiment and to further assess the potential of CDK inhibitor drugs to translate to clinical entities we used an *i.t.* aspiration model to mimic the delivery of standard inhaled therapies. This allowed us to decrease the total dose delivered to 10mg/kg given as per the schema shown in figure 7.1.

Figure 7.1: *In vivo* experimental plan for assessment of inhibition of CDKs 7 and 9 in acute phase of bleomycin lung injury



Mice treated with bleomycin at day 0 develop a significant neutrophil-predominant infiltrate after 24h. This usually persists to 7 days (figures 7.2A, C and 7.3) and is associated with significant damage to the epithelium which eventually results in collagen deposition and fibrosis. Mice treated with DRB or R-roscovitine on days 2 and 4 clear the bleomycin-induced multifocal inflammatory cell infiltration from the alveoli, peri-bronchiolar and peri-vascular areas by day 7 and their lungs remain architecturally intact with only minimal histological evidence of inflammation (figure 7.2B and D). This is in contrast to untreated mice where neutrophils persist and significant architectural disturbance occurs resulting in fibrosis.

Mice subjected to bleomycin injury develop significant BAL neutrophilia (figure 7.3) and additionally a measurable increase in BAL protein (figure 7.4) suggestive of inflammatory injury. By contrast mice subjected to bleomycin injury but treated with the CDK7/9- specific inhibitor DRB or R-roscovitine have less neutrophils in BAL with no detrimental effect on macrophage numbers (figure 7.3). Additionally, there is significantly less protein in BAL of these mice compared to vehicle control (figure 7.4). There is an apparent trend towards less LDH in BAL of treated mice that is statistically insignificant (figure 7.4).

Figure 7.2: The CDK 7/9-specific inhibitor, DRB resolves acute neutrophil-dominant inflammation

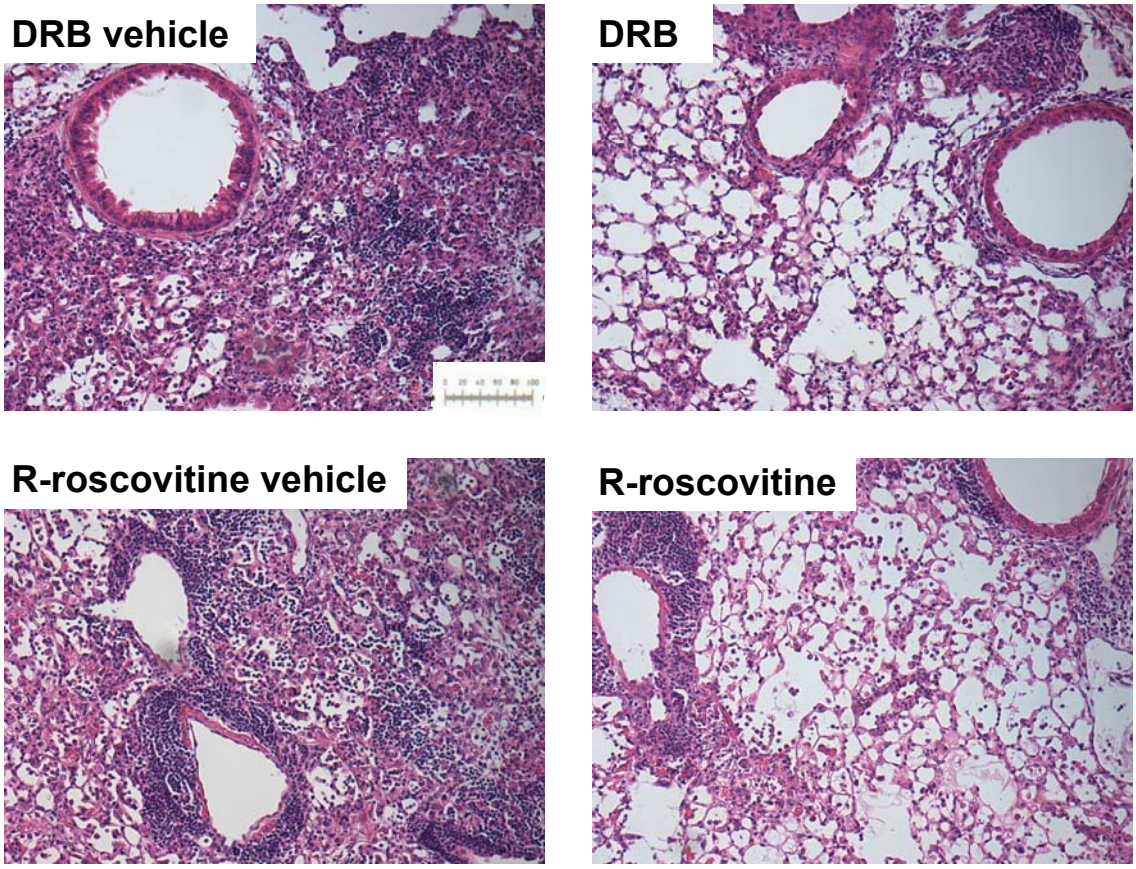


Figure 7.2: Representative histology from Bleomycin acute lung injury model experiment. Images are at magnification x100 (Size bar μm).

Figure 7.3: DRB reduces BAL neutrophil count with no detrimental effect on macrophage numbers

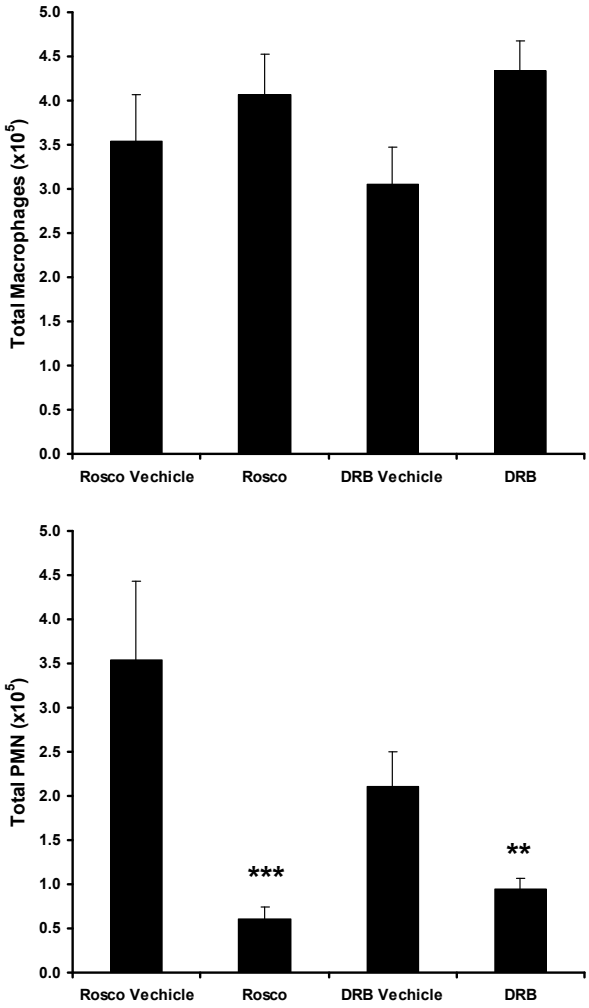


*Figure 7.3: Differential white cell counts from broncho-alveolar lavage of mice subjected to bleomycin injury and treated according to the schedule in figure 7.1. Statistically significant difference compared to vehicle control shown as ** $p<0.01$ or *** $p<0.001$ by ANOVA with a Student Newman-Keuls multiple comparison post hoc test with a 95% confidence interval.*

Figure 7.4: DRB reduces protein levels with no significant effect on LDH levels

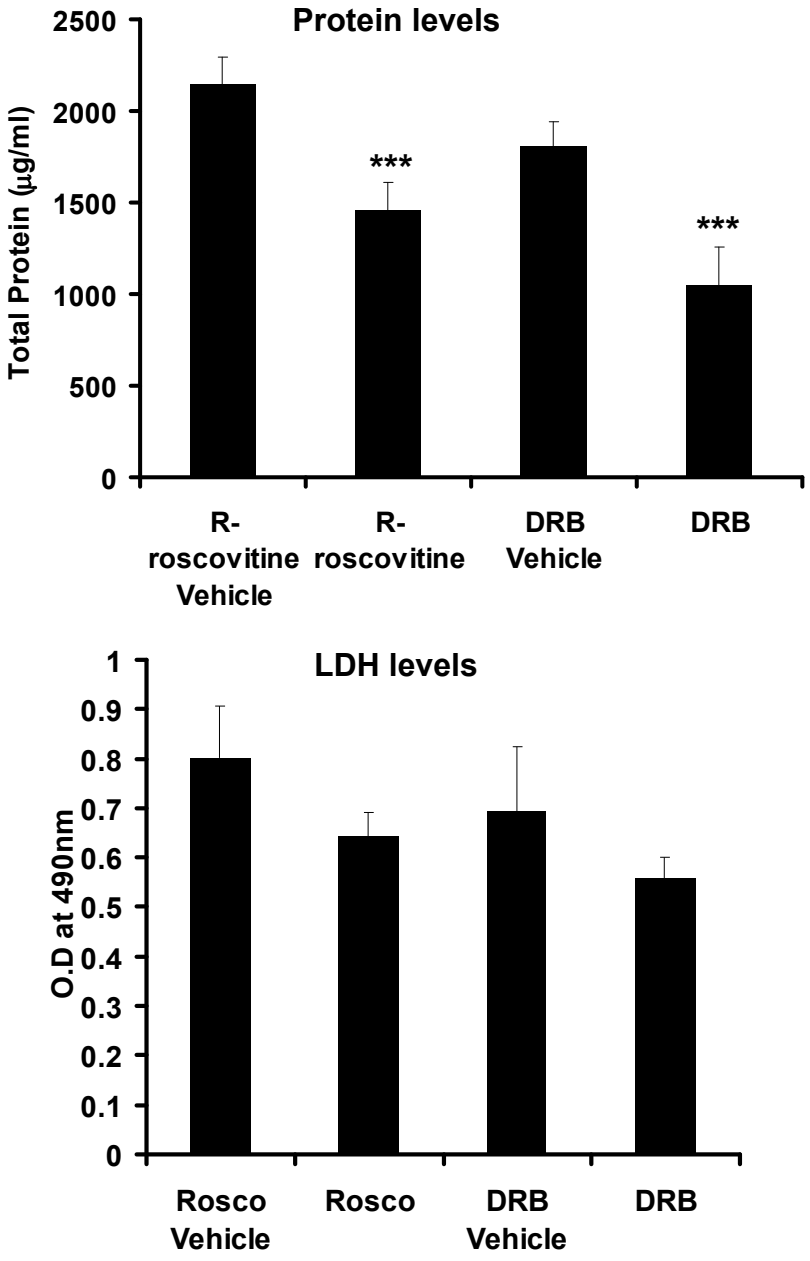


*Figure 7.4: Total protein and LDH levels from BAL of mice subjected to bleomycin injury and treated according to the schedule in figure 7.1 Statistically significant difference compared to control shown as *** $p<0.001$ by ANOVA with a Student Newman-Keuls multiple comparison post hoc test with a 95% confidence interval. No statistically significant difference in LDH levels.*

7.2 Discussion of Chapter 7

We have previously shown that a CDK inhibitor drug, R-roscovitine, can resolve bleomycin-induced lung injury by promotion of neutrophil apoptosis. We were keen to incisively investigate the mechanism responsible for this effect by knocking down specific, individual CDKs in neutrophils *in vivo*. We had planned to achieve this by producing dominant negative CDK proteins coupled to the tat cell penetrating peptide. The production of these proteins encountered various difficulties (we are still confident we will be able to produce these proteins) and we therefore utilised the CDK7 and 9-specific pharmacological inhibitor DRB. The experiment we performed (as detailed in the schematic in figure 7.1) aimed to compare the impact of DRB and R-roscovitine (CDK2, 5, 7 and 9 specific) on the early (first week) neutrophil-dominant inflammation induced in mouse lungs by *i.t.* administration of bleomycin. Additionally we attempted a local delivery of CDK inhibitor drugs by *i.t.* administration in an attempt to mimic the aerosol administration of inhaled/nebulised therapy in use in respiratory disease such as COPD/asthma. *I.t.* administration allowed us to reduce the dose of drug given to the animals ten-fold (100mg/kg to 10mg/kg). We expect that local delivery of a reduced drug-dose may help to attenuate some of the side-effects encountered in humans when these drugs are used as cancer therapeutics.

BAL and lung tissue were retrieved and cell-counts and histology revealed effective neutrophil depletion in BAL and tissue. Macrophage numbers remained constant in BAL. We are confident that neutrophil apoptosis is induced *in vivo* rather than, for example, an inhibition of neutrophil recruitment because neutrophil-dominant inflammation is already established by the time we administer CDK inhibitor drugs. In addition we observe neutrophil debris and apoptotic bodies within macrophages in mouse BAL and tissue from animals treated with CDK inhibitor drugs.

Additionally, there is evidence of resolution of bleomycin-induced inflammatory injury by CDK inhibitor drugs in the form of reduced protein leak measured in BAL. No

significant difference in measured LDH levels was detected though there was a trend towards less LDH in in CDK inhibitor treated mice. This probably reflects the use of a model of established neutrophilic inflammation where a degree of cell damage has already occurred by the time we institute treatment. We would hypothesise that levels of LDH would fall at later time-points as resolution is allowed to progress whereas they might be expected to be maintained in untreated animals.

Summary of Chapter 7

1. **The CDK7 and 9-specific inhibitor, DRB can resolve bleomycin-induced, neutrophil-dominant lung injury.**
2. **CDK7 and 9 are potential targets for pro-resolution of inflammation therapeutics in the treatment of lung disease**
3. **Macrophage numbers are not adversely affected by CDK inhibitor drug therapy**
4. **Local *(i.t.)* administration of CDK inhibitor drugs is an effective strategy for drug-delivery**

8 General Discussion

Inflammation is recognised as central to the pathogenesis of a number of disease entities including: cancer, atherosclerosis, arthritis, glomerulonephritides, asthma and smoking-related lung disease (Hallett et al. 2008b;Rossi et al. 2007;Serhan et al. 2007). Consequently, it is an important target for the design of new therapeutics. These therapeutics have traditionally targeted the initiation and propagation phases of inflammation. Important examples include NSAIDs which target COX the enzyme that drives production of prostaglandins which are key mediators in the early stages of inflammation and glucocorticosteroids which work through a variety of effects at the molecular level including enhancement of anti-inflammatory and repression of pro-inflammatory gene transcription (Newton 2000). They are very successful at treating eosinophil-dominant inflammatory disease such as asthma while they have had more modest (possibly arguable) success with neutrophil-dominant disease. It is now known that glucocorticoids promote eosinophil apoptosis but actually prolong neutrophil survival (Heasman et al. 2003;Sivertson et al. 2007;Ward et al. 1999). The latter effect is, presumably, partially compensated by glucocorticoid-mediated, enhanced macrophage phagocytosis of apoptotic neutrophils (Heasman et al. 2003). Additionally, designer anti-cytokine therapies like the anti-TNF-α agents have aimed to remove the main constituents of ongoing inflammation. More recently, a movement towards specifically targeting the resolution phase of inflammation has been evident (Serhan et al. 2007). The key determinants of appropriate resolution of inflammation include removal of the inciting stimulus, controlled death (usually by apoptosis) of inflammatory leucocytes and removal of dead cells and debris by phagocytic cells. Indeed, the compound RX-10045 (a therapeutic based on the lipid mediator resolvin) which has multiple pro-resolution properties is likely to translate into routine clinical usage in the near future (www.resolvyx.com).

The death by apoptosis of the early-responding and most abundant inflammatory leucocyte, the neutrophil, has been identified as a key step in the resolution of

inflammation (Hallett et al. 2008b;Haslett 1999). It is a key step not just because it removes a potentially injurious cell population (Kobayashi et al. 2003;Koedel et al. 2009) but because a pro-resolution signal is sent by both the process of apoptosis (Perretti et al. 2004;Scannell et al. 2007a;Voll et al. 1997) and the phagocytic removal of apoptotic cells (Fadok et al. 1998;Voll et al. 1997). The proof of concept experiment, delivery of apoptotic cells to mice subjected to LPS-induced inflammation as a pro-resolution therapeutic powerfully demonstrated the potential for treatment of inflammation driven disease (Huynh et al. 2002;Ren et al. 2008).

In keeping with this finding CDK inhibitor drugs were shown to drive neutrophil apoptosis and to promote resolution of inflammation (Rossi et al. 2006). These drugs were developed as anti-cancer therapeutics as they inhibit CDKs which are master-regulators of the cell-cycle (Knockaert et al. 2002;Senderowicz 2003). This mechanism of action was not directly translatable to neutrophils as they are non-proliferating, terminally differentiated cells. Indeed, the only other terminally differentiated cells treated with these drugs, neuronal cells, have been shown to respond with enhanced longevity (Blondel et al. 2007). The over-arching aim of this thesis is to determine the mechanism by which cyclin-dependent kinase inhibitor drugs promote neutrophil apoptosis.

The first results chapter (chapter 3) addresses the pharmacokinetics of CDK inhibitor-driven neutrophil apoptosis and investigates established molecular mechanisms of neutrophil apoptosis in order to provide a framework for further characterisation of underlying mechanism. In this chapter I have determined the concentration of CDK inhibitor drug which will be investigated in the remainder of the thesis and have characterised the time-line of neutrophil death both constitutively and in the presence of CDK inhibitor drugs. This chapter shows conclusively that apoptosis is the primary mode of cell death induced by CDK inhibitor drugs and that necrosis only occurs after a time-delay as a secondary process. These findings are clearly demonstrated in figures 3.1-3.3. I have implicated the caspase machinery in CDK inhibitor driven neutrophil

death and have shown that a key early step in this process is the loss of mitochondrial membrane integrity. Surprisingly given our earlier work which showed that direct inhibition of the key neutrophil survival signalling pathway NF-κB was not applicable to the CDK inhibitor drug effect (Leitch et al. 2010b), I show that early apoptosis driven by TNF-α is enhanced by CDK inhibition (figure 3.8). This finding along with loss of mitochondrial membrane integrity (figure 3.13), similar kinetics of apoptosis to protein synthesis inhibitor drugs (figure 3.5) and relevant findings in the literature implicated the down-regulation of a survival protein in the mechanism of apoptosis.

The fourth chapter of this thesis pursues the hypothesis that a key survival protein is down-regulated in order to allow neutrophil apoptosis to proceed. It is already established in the literature that the pre-eminent neutrophil survival protein is the bcl-2 homologue Mcl-1 (Dzhagalov et al. 2007a;Edwards et al. 2004b). In this chapter it is demonstrated that Mcl-1 is down-regulated by CDK inhibitor drugs even in the presence of inflammatory pro-survival signals. These data complement the chapter 3 finding that the enhancement of neutrophil longevity by pro-survival agents is overcome by CDK inhibitor drugs. In the reverse of this experiment preservation of Mcl-1 by pharmacological means (proteasome inhibition) delays the onset of CDK inhibitor drug induced apoptosis. It is shown that the balance of bcl-2 homologue signalling is tipped towards apoptosis by the persistence of key pro-apoptotic homologues (especially Bim) even in the presence of CDK inhibitor drugs. PCR for MCL-1 demonstrates that it is down-regulated at the level of transcription by CDK inhibitor drugs. This finding coupled with the similar kinetics of apoptosis of protein synthesis inhibitor drugs and pertinent findings in the literature related to the transcriptional roles of CDKs 2, 7 and 9 instigated the investigation of an effect of CDK inhibitor drugs at the level of transcription in chapter 5.

This chapter covers the identification and characterisation of the sub-cellular distribution of CDKs and binding partners within neutrophil granulocytes. CDKs are known to be subject to complex layers of regulation. This includes activation by binding partner

cyclins, inhibition by endogenous CDK inhibitors, phosphorylation by activating CDKs and other kinases, inhibitory binding and sequestration (7SK etc) and nucleo-cytoplasmic shuttling. Initially the CDKs and cyclin binding partners known to fall within the well-characterised pharmacological specificity of R-roscovitine (the most extensively studied CDK inhibitor drug) are identified at protein and gene expression levels. The data generated in Chapter 2 and the expression data with regard to CDKs 7 and 9 suggest that these may be key targets because of their known role in the process of transcription. These CDKs regulate transcription by phosphorylation of the C-terminal domain of RNA polymerase II. CDK7 phosphorylation at the key serine 2 residue allows transcription initiation to proceed while CDK9 phosphorylation at serine 2 and 5 allows transcript elongation. This is the first time the fundamental regulation of the key enzyme of eukaryotic mRNA transcription RNA polymerase II has been investigated in neutrophils. This provides evidence that the most important target of the CDK inhibitor R-roscovitine in the promotion of neutrophil apoptosis is the inhibition of the transcriptional role of CDKs 7 and 9. In order to further investigate this novel finding an assessment of the neutrophil transcriptome is performed at the constitutive level and in the presence of R-roscovitine. The gene expression data support the idea of CDK inhibition down-regulating transcription through effects on RNA polymerase II. In addition it appears that the transcripts regulated by CDK7 and 9 are signalling responsive, rapidly up/down-regulated genes many of which have key roles in transcription, apoptosis and inflammation. Additionally, this experiment increases the weight of evidence for a critical differential at the level of mRNA and protein between Mcl-1 and Bim that would shift neutrophil fate towards apoptosis.

In Chapter 6 an attempt is made to devise a molecular biology strategy that would allow specific targeting of CDKs 7 and 9 in neutrophils to establish an alternative to pharmacological knockdown and to support the hypotheses and results generated in chapters 1-3. After consideration of the currently available strategies for targeted knockdown of proteins an attempt is made to develop a cell-delivery peptide based experiment where dominant-negative CDKs would be introduced into neutrophils. This

strategy was the most appealing because of the time-constraints imposed by limited neutrophil life-span and the known (and demonstrated) toxicity of lipid-based delivery agents. The production of HIV tat linked dominant negative CDKs proved a time-consuming and difficult process. It was considerably hampered by the difficulties involved in isolating functional, pure protein from insoluble pellet fractions. It remains a work in progress.

In Chapter 7 a more specific pharmacological inhibitor of CDKs 7 and 9 is shown to resolve neutrophil-dominant inflammation in the murine bleomycin lung injury model. This finding supports the *in vitro* data generated in chapters 1-3 that suggest the most important targets of the CDK inhibitor R-roscovitine are CDKs 7 and 9. In addition the use of *i.t.* administration demonstrates that a ten-fold reduction in dose is possible by a delivery method that is easily translatable to human therapeutics (e.g., inhalers, nebuliser).

This thesis has added to the published literature on apoptotic mechanisms pertinent to neutrophil granulocytes by highlighting the importance of the anti-apoptotic bcl-2 homologue survival protein Mcl-1 and the pro-apoptotic bcl-2 homologue Bim. This thesis increases the weight of evidence suggesting not only that neutrophil granulocytes are capable of *de novo* transcription but that this facility is indispensable to both constitutive and stimulated neutrophil survival. It is the first work to characterise the CDK machinery involved in the regulation of transcription by RNA polymerase II and to identify the presence and modulation by phosphorylation of this important holoenzyme in neutrophil granulocytes. In addition this thesis highlights the potential for selective modulation of transcriptional machinery towards the promotion of pro-resolution phenotypes not just in neutrophils but across a range of cell types. In particular the thesis shows how rapidly signal-responsive, upregulated genes involved in immune response, apoptosis and transcription can be down-regulated by inhibition of components of the transcriptional machinery. The thesis confirms that such specific inhibition of transcriptional machinery can promote resolution of inflammation in a murine model

without deleterious off-target effects. In addition, the specific transcriptional effects demonstrated may explain the limited side effects associated with systemic delivery of CDK inhibitor drugs. Regardless, efficacious *i.t.* delivery in our murine experiments suggests that there is potential for the local delivery of CDK inhibitor drugs to sites of inflammation including the lungs via inhalation.

The potential future application of CDK inhibitor drugs to the treatment of human inflammatory disease is a realistic prospect. The suitability of these agents for translation is in part shown by data in this thesis but also by the known side-effect profile that has emerged from their use in human clinical trials as cancer therapeutics. Our group has begun an experimental medicine trial of a CDK inhibitor drug in patients with IPF which will investigate multiple parameters of anti-inflammatory response including: reduction of BAL neutrophilia and change in PET signal as well as clinical parameters such as pulmonary function tests and exacerbation frequency. It is important in the assessment of potential anti-inflammatory agents that the anti-inflammatory/pro-resolution effects observed in animal models are achieved in humans (e.g., reduction in BAL neutrophil count) as well as assessing traditional measures of disease activity to allow a correct evaluation of the agent studied (Katharine et al. 2009). Other conditions that might benefit from clinical trial of these drugs include vasculitides (Gomez-Puerta et al. 2009) and resistant eosinophilic disorders (Duffin et al. 2009;Roufosse et al. 2010) where there is multi-system granulocyte-dominant inflammation. In these conditions immunosuppressive agents are already established as gold-standard therapies. A more controversial but potentially extremely rewarding use of these agents might be in the treatment of severe infections unresponsive to antibiotics and especially where ARDS supervenes. There is a precedent for the use of a CDK inhibitor drug as an apoptosis-inducing agent in an infective condition provided by the study of bacterial meningitis by Koedel et al where R-roscovitine and an antibiotic were shown to promote better resolution of pathology than an antibiotic alone (Koedel et al. 2009).

This thesis highlights the difficulty inherent in the application of 'standard' molecular biology approaches to *in vitro* neutrophil experimentation. Further work will be required to successfully develop and utilise cell-permeable peptide delivery agents in the investigation of potential resolution of inflammation targets. In addition the development of lentiviral/siRNA tools for the manipulation of stimulated and hence longer-lived neutrophils may be a succesful alternative strategy.

Further experiments that will enhance the work contained within this thesis will include:

1. The application of models of inflammation to the myeloid MCL1-/- mouse. The MCL-1-/- mouse is relatively neutropenic and those neutrophils that are produced are short-lived. These experiments will determine the importance of Mcl-1 to neutrophils under inflammatory conditions. They will also afford the opportunity to dissect out the role of long-lived, activated neutrophils in causing inflammatory damage.
2. An assessment of CDK inhibition on macrophage signalling and on the macrophage transcriptome using a bioinformatics based approach
3. An assessment of CDK inhibition on fibroblast signalling and on the fibroblast transcriptome with a view to the investigation of models of aberrant wound healing/fibrogenesis (e.g. late-stage bleomycin lung injury)
4. Alternative methods for generation of CDK-dn peptides- including mammalian cell based production and alternative isolation tags
5. Alternative molecular biology techniques including lentiviral, siRNA transduction of stimulated cells

9 Concluding remarks

This thesis identifies the key targets of the CDK inhibitor drugs to be CDKs 7 and 9 which are responsible for the regulation of specific genes transcribed by RNA polymerase II, in particular the survival protein Mcl-1 which is critical for neutrophil survival (figure 8). Consequently, the thesis emphasises the importance of neutrophil apoptosis to the resolution of inflammation and supports the therapeutic trial of CDK inhibitor drugs in inflammatory disease. The potential for translation of these drugs or other targets identified in their investigation is exemplified by an experimental medicine trial of a CDK inhibitor drug for the treatment of patients with idiopathic pulmonary fibrosis which is in progress in our institute. The data in this thesis would support further application to neutrophil-dominant diseases that would include: ALI, exacerbation of IPF, pneumonia and vasculitides.

Figure 8: Model of CDK inhibitor drug inhibition of neutrophil transcription leading to apoptosis

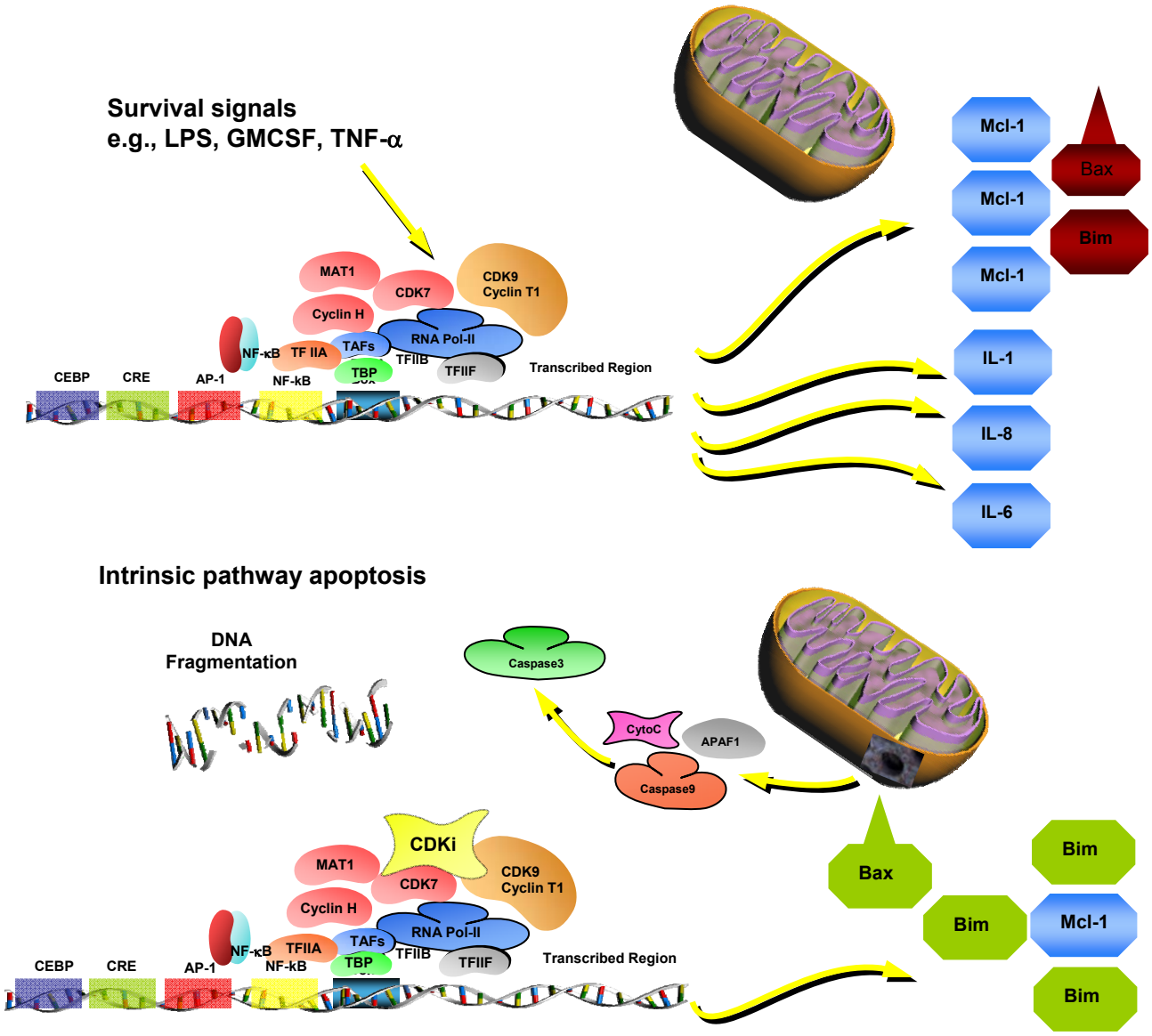


Figure 8: Survival signals promote CDK7 and 9 -dependent transcription of inflammatory cytokines and the survival protein, Mcl-1. Mcl-1 sequesters Bim in an inactivated state preventing apoptosis. When CDK inhibitor (CDKi) is present Bim is upregulated, Mcl-1 is no longer transcribed and Bim is able to activate Bax which leads to intrinsic pathway apoptosis with mitochondrial outer membrane permeabilisation in the neutrophil granulocyte.

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