UNIVERSITY OF LIVERPOOL

Regulation of Neutrophil Function by NAMPT

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

KATE JULIA ROBERTS

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I declare that this thesis entitled:

“Regulation of Neutrophil Function by NAMPT”

is entirely my own work except where indicated in the text.

Candidate: **Kate Julia Roberts**

Supervisors: **Professor S.W. Edwards**

Institute of Integrative Biology

University of Liverpool

Professor R. J. Moots

Institute of Aging and Chronic Disease

University of Liverpool

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ABSTRACT

Neutrophils have capacity to cause tissue damage in chronic inflammatory disease. In rheumatoid arthritis (RA) they infiltrate joints, secrete proteases and reactive oxygen species (ROS), and express cytokines. RA neutrophils express NAMPT which is a highly conserved, pleiotropic protein, that catalyses the rate-limiting step of the NAD salvage pathway, but also has cytokine-like activity. NAMPT is elevated in inflammation and exerts pro-inflammatory effects on neutrophils. The aim of this research was to determine the role of NAMPT, as a signalling molecule and as an enzyme, in regulating neutrophil activities of pathological importance.

Neutrophils were isolated from the blood of healthy donors and either stimulated with NAMPT or else NAMPT was inhibited (with FK866), in the presence and absence of pro-inflammatory cytokines *in vitro*. Assays for specific neutrophil functions such as production of ROS, bacterial killing and apoptosis were performed, and expression of cytokines was also examined. Transcriptome sequencing of neutrophils treated with FK866 and TNFα in combination, was carried out to investigate the wider impact of NAMPT inhibition on neutrophil gene expression. NAMPT expression was also examined in control, cytokine treated and RA patient neutrophils.

NAMPT had little capacity to prime neutrophils, although it did delay neutrophil apoptosis and stabilise the anti-apoptotic protein Mcl-1. NAMPT inhibition in neutrophils, depleted NAD(P)/H and had effects on neutrophil functions and gene expression. Notably, FK866 decreased ROS production but did not affect the ability of neutrophils to kill bacteria. NAMPT-inhibited neutrophils exhibited decreased activation of signalling pathways and a diminished response to cytokines; transcriptome analysis showed that FK866 decreased TNFα-induced expression of many genes. NAMPT expression was not dynamically regulated in control neutrophils, but in RA patient neutrophils, NAMPT mRNA correlated with TNFα expression in a cohort of patients, and NAMPT protein was elevated in synovial fluid neutrophils compared to those from paired blood.

Thus, NAMPT is elevated in activated inflammatory neutrophils and correlates with other markers of inflammation in RA, suggesting that it may be a marker of inflammation in these cells. Also, as a NAD biosynthetic enzyme, NAMPT regulates neutrophil functions and gene products central to RA disease pathology, without affecting bacterial killing capacity. This suggests that inhibition of NAMPT may potentially have the capacity to decrease neutrophil mediated tissue-damage observed in chronic inflammation, without adversely compromising host defence, and thus may be a future treatment option for diseases such as RA.

PUBLICATIONS AND PRESENTATIONS

Publications

Roberts, K. J., Vasieva, O., Moots, R. J., and Edwards, S. W. (2012) Inhibition of Pre-B Cell Colony-Enhancing Factor (PBEF/NAMPT/ Visfatin) Decreases the Ability of Human Neutrophils to Generate Reactive Oxidants, but does not Impair Bacterial Killing. *Submitted to the Journal of Immunology.*

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Oral Presentations

Roberts, K. J., Moots R. J., and Edwards, S. W. Inhibition of NAMPT Decreases the Ability of Human Neutrophils to Generate Reactive Oxidants, but does not Impair Bacterial Killing. Presented at the British Society for Rheumatology Conference, Glasgow in May 2012.

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ABBREVIATIONS

·OH	Hydroxyl free radical
6PGDL	6-phosphogluconolatone
AP-1	Activator protein-1
Apaf-1	Apoptotic protease-activating factor-1
APC	Allophycocyanin
APS	Ammonium persulphate
ARDS	Acute respiratory distress syndrome
ART	ADP-ribose transferase
Bad	B-cell leukaemia-2 associated death promoter protein
Bak	B-cell leukaemia-2 homologous antagonist/killer protein
Bax	B-cell leukaemia-2 associated protein-X
BCA	Bicinchoninic acid
Bcl-2	B-cell leukaemia-2 protein
Bcl-X_L	B-cell lymphoma-extra large protein
Bfl-1/A1	B-cell leukaemia-2 related protein A1
BGI	Beijing Genomics Institute
BH	B-cell leukaemia-2 homology domain
Bid	BH3 interacting domain death antagonist protein
Bik	BCL2-interacting killer (apoptosis-inducing)
Bim	B-cell leukaemia-2 like protein-11
BimL	B-cell leukaemia-2 like protein-11 (isoform-6)
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
BSA	Bovine serum albumin
cADP	Cyclic ADP
Caspase	Cysteine-aspartic acid protease
Caspase	Cysteine-aspartic proteases
CB	Cytochalasin B
CCL	Chemokine (C-C motif) ligand

CCP	Cyclic citrullinated peptide
CCP	Cyclic citrullinated peptide
CCR	C-C chemokine receptor
Cfu	Colony-forming units
CGD	Chronic granulomatous disease
CGD	Chronic granulomatous disease
CHX	Cycloheximide
CIA	Collagen-induced arthritis
cIAP	Cellular inhibitor of apoptosis
COPD	Chronic obstructive pulmonary disease
CORE	Committe for research ethics
CR	Complement receptor
CRADD	Caspase-2 and RIPK1 domain containing adaptor with death domain
CRP	C-reactive protein
CYBA	Cytochrome b alpha
CYBB	Cytochrome b beta
DAPK1	Death-associated protein kinase 1
DAS	Disease activity score
ddH_2O	Double-distilled water
DEPC	Dethylpyrocarbonate
DISC	Death-inducing signalling complex
DMARD	Disease modifying anti-rheumatic drug
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetracetic acid
eNAMPT	Extracellular NAMPT
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate
EULAR	European League against Rheumatism

FAD	Flavin adenine dinucleotide
FADD	Fas-associated death domain-containing protein
FasL	Fas ligand
FcγR	Fcγ receptor
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
G6P	Glucose-6-phosphate
G6PDH	G6P dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulating factor
GPI	Glycosyl-phosphatidylinositol
GROβ	Growth-regulated oncogene β
GSK3	Glycogen synthase kinase-3
GTPase	Guanosine triphosphatase
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
HDAC	Histone deacetylases
HIF1α	Hypoxia-inducible factor 1α
HMP	Hexose monophosphate
HOCl	Hypochlorous acid
HRP	Horseradish peroxidise
HSA	Human serum albumin
ICAM	Intercellular adhesion molecule
IFNγ	Interferon-γ
IgG	Immunoglobulin G
IL	Interleukin
IL-1Ra	IL-1 receptor agonist
iNAMPT	Intracellular NAMPT
iNOS	Inducible nitric oxide synthase
IPA	Ingenuity pathway analysis
JAK	Janus kinase

JAM	Junctional adhesion molecule
JIA	Juvenile idiopathic arthritis
JNK	C-Jun N-terminal kinase
LF	Lactoferrin
LPS	Lipopolysaccharide
MAC-1	Macrophage-1 antigen
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia 1
MEK1	Mitogen-activated protein kinase 1
MEKK1	Mitogen-activated protein kinase kinase 1
MHCII	Class II major histocompatibility complex
MIP1	Macrophage inflammatory protein 1
MMP	Matrix-metalloproteinase
MPO	Myeloperoxidase
Na	Nicotinic acid
NaADP	Nicotinate adenine dinucleotide phosphate
NAD	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADK	Nicotinamide adenine dinucleotide kinase
NADP	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NAM	Nicotinamide
NAMPT	Nicotinamide phosphoribosyltransferase
NaPRTase	Nicotinic acid phosphoribosyltransferase
NCF	Neutrophil cytosolic factor
NF-1	Nuclear factor-1
NF-κB	Nuclear factor κB
NIK	NFκB-inducing kinase
NMN	Nicotinamide mononucleotide
NMNAT	Nicotinamide mononucleotide adenylyl transferase

NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
PAMP	Pathogen-associated molecular pattern
PARP	Poly-ADP-ribose polymerases
PBEF	Pre-B-cell colony stimulating factor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PECAM	Platelet endothelial cell adhesion molecule
PET	Polyethylene Terephthalate
PFA	Paraformaldehyde
PHOX	Phagocyte oxidase
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PRPP	5-phosphoribosyl-pyrophosphate
PRT	Phosphoribosyltransferase
PVDF	Polyvinylidene fluoride
QaPRTase	Quinolinic acid PRT
RA	Rheumatoid arthritis
Rac1/2	Ras-related C3 botulinum toxin substrate 1/2
RANKL	Receptor activator of nuclear factor kappa-B ligand
RF	Rheumatoid factor
RhoGDI	Rho guanine nucleotide dissociation inhibitor
RIN	RNA integrity number
ROS	Reactive oxygen species
RPKM	Reads per kilobase-pair per million mapped reads
SDS	Sodium dodecyl sulphate
SF	Synovial fluid
sIL-6R	Soluble IL-6 receptor
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphisms

SP-1	Specificity Protein-1
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptor
TNFAIP	Tumor necrosis factor alpha-induced protein
TNFR	Tumour necrosis factor receptor
TNFRSF	Tumor necrosis factor receptor superfamily
TNFα	Tumour necrosis factor α
TRADD	TNFR-associated death domain-containing proteins
UTR	Untranslated region

CHAPTER 1: Introduction

1.1 Project Overview

Neutrophils are an essential component of the innate immune system; they contain an arsenal of anti-microbial proteins and oxidants which are employed to kill invading pathogens [1]. Neutrophils however act as a double-edged sword, as this anti-microbial arsenal is also capable of causing host tissue damage if neutrophil function and apoptosis are not appropriately controlled [2]. Regulation is achieved by the two-step activation of neutrophils; initial priming by immune mediators followed by subsequent full activation, and also by the short half-life and constitutive apoptosis of these cells [3–5]. Neutrophil apoptosis is transiently delayed to allow response to an immune challenge. However, dysregulation of neutrophil activation and apoptosis is a key feature of a number of inflammatory diseases, including the inflammatory joint disease rheumatoid arthritis (RA) [2,6]. Neutrophils are primed by exposure to increased levels of circulating cytokines in this disease, and migrate towards the high local concentration of chemoattractants in the inflamed rheumatoid joint. Neutrophils infiltrate the normally acellular rheumatoid joint in large numbers, and are by far the most abundant immune cell present [7,8]. Once in the joint space, primed neutrophils are stimulated to release cytotoxic proteins and reactive oxygen species (ROS), mainly by immune complexes of autoantibodies and antigen [9]. These complexes can

also be found embedded in the pannus of proliferative tissue that is the site of bone and cartilage degradation in this disease [10]. These neutrophil products cause a significant amount of tissue damage at these sites, as neutrophil apoptosis is delayed for up to several days following migration into the joint [2,11–13].

Rheumatoid arthritis is a heterogenous disease and its etiology is incompletely understood. As such, the patient response to the various treatments available is also varied [14]. As RA is progressively degenerative, it is important to establish patients on a successful treatment regimen as soon as possible, and although many patients do respond well to conventional therapies, a number remain refractory to treatment. As such it is of interest in the field to identify novel biomarkers of disease that may serve as diagnostic predictors of response to treatment or lead to development of new drug targets [14–16]. Neutrophils are an attractive target in RA because they are the main cell type responsible for causing tissue damage in the disease [11].

It is now appreciated that neutrophils also contribute to the progression of inflammation via expression of cytokines and chemokines; the expression profile of an activated neutrophil is very different from that of a resting cell [17]. A high-level screen of the neutrophil transcriptome in a number of inflammatory diseases (RA, Beçet's disease and scleroderma) was carried out in-house to identify disease-specific neutrophil biomarkers of

activation. Notably, high expression of the transcript for NAMPT was observed across all diseases. NAMPT has been previously identified as correlating with inflammation and inflammatory disease markers [18], and is proposed to exert a number of pro-inflammatory effects in a number of cell types [19–24]. It is a ubiquitously expressed molecule with a number of described functions [23,25]. It has been established that NAMPT is a nicotinamide phosphoribosyltransferase enzyme that catalyses the rate-limiting step in the nicotinamide adenine dinucleotide (NAD) salvage pathway. NAD is an essential metabolic co-factor and is required for a number of oxidation reactions [26–30]. NAMPT has also been described as a cytokine-like molecule, distinct from its enzyme activity, and more controversially has been described as an insulin mimetic adipokine [20,22,23,31,32]. Inhibition of the enzyme function of NAMPT has shown promise for the alleviation of inflammation and the symptoms of inflammatory disease in mouse models of inflammatory arthritis, and it has also been reported to exert a number of anti-inflammatory effects on immune cells *in vitro* [33–35].

The high level of NAMPT expressed by neutrophils from patients with inflammatory diseases, and its correlation with disease activity, suggest that it is an important molecule in the regulation of the inflammatory process. The work in this thesis investigated the role of NAMPT in a variety of neutrophil functions important in inflammation, and on

neutrophil gene expression. The NAMPT inhibitor, FK866 was used to investigate the role of NAMPT and NAD production on these neutrophil properties. RA was used as a model of inflammatory disease. The aim of this research was to achieve a better understanding of the role of NAMPT in neutrophil function in inflammation, and to determine how therapeutic NAMPT inhibition would affect the ability of these cells to carry out their role in host defence.

1.2 The Neutrophil

1.2.1 Overview of neutrophil function

Neutrophils are key components of the innate immune system and represent the first line of defence against bacterial and fungal infection [36]. Neutrophils are produced in the bone marrow at a rate of 5 x 10^{10} – 10 x 10^{10} cells/day and circulate in the blood in an inactive state; they are the most common white blood cell, found at a concentration of 3-5 x 10^{6} cells per mL of blood [11,37]. Marginated pools of neutrophils are also found within specific tissues such as the spleen and liver and at the site of production in the bone marrow [37]. Neutrophil homeostasis in the circulation is achieved by control of production and release but also by apoptosis; neutrophils constitutively undergo apoptosis and have a relatively short half-life [38]. Estimates for this half-life in the circulation vary from 6-18 h, but recent evidence suggests that this may be an underestimate [4,5,39]. Upon initial exposure to immune agonists or cytokines,

inactive circulating neutrophils undergo a series of rapid changes, known collectively as priming, and their apoptosis is transiently-delayed. Priming enables a more effective response to an invading pathogen as it rapidly increases the number and/or affinity of plasma membrane receptors for immune agonists and overall enhances the capacity for bacterial killing [1,3,40]. Primed neutrophils also produce cytokines which activate and recruit further neutrophils and other immune cells [41,42]. The dynamic regulation of neutrophil membrane receptors facilitates transmigration along a chemotactic gradient towards the site of infection, binding to the blood vessel endothelium and migration into the tissue. Upon encountering a pathogen, membrane receptors facilitate recognition and binding, and the pathogen is engulfed by phagocytosis (Fig. 1.1). Neutrophils then employ a number of potent bactericidal mechanisms to destroy the engulfed pathogen. Firstly, neutrophils contain membrane bound granules which hold a variety of proteases, and following phagocytosis these granules fuse with the phagocytic vesicle and release the cytotoxic granule proteases into it. This occurs concurrently with the second major antimicrobial mechanism, production of reactive oxygen species (ROS). ROS are produced by reduction of molecular O_2 during the respiratory burst, which is catalysed by the membrane bound NADPH oxidase complex [43]. These granule proteases and ROS act in concert to destroy the pathogen in the phagocytic vesicle [44]. Following pathogen destruction, the neutrophil undergoes controlled apoptosis to prevent release of its cytotoxic contents

into the extracellular milieu [5,45]. Apoptotic neutrophils are removed by other phagocytic immune cells such as macrophages, to facilitate resolution of inflammation [46]. An overview of this process is shown in Figure 1.1, and each stage is discussed in more detail in the following sections.

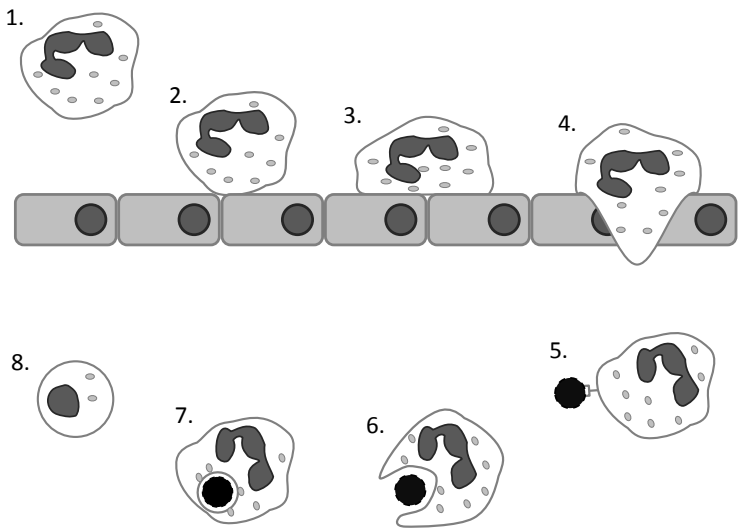

FIGURE 1.1: Overview of neutrophil function in immune response. Peripheral blood neutrophils (1.) become tethered to the blood vessel endothelial cell lining and roll along the surface (2.) The neutrophil then firmly adheres to the vessel wall (3.) and squeezes between gaps in the neighbouring endothelial cells to enter the tissue (4.). The neutrophil travels along a chemotactic gradient towards the site of infection, and recognises and binds pathogens by virtue of cell surface receptors (5.). The neutrophil engulfs the pathogen by phagocytosis (6.) and destroys it by releasing granule proteases and ROS into the phagocytic vesicle (7.). The neutrophil then undergoes controlled apoptosis (8.).

In contrast to the crucial role the neutrophil plays in host defence, dysregulation of neutrophil function is also implicated in numerous human diseases. If neutrophil function is not tightly regulated, this cell has great capacity to cause tissue damage due to the cytotoxic products it contains. Destructive neutrophil products are major contributors to tissue damage in inflammatory diseases such as rheumatoid arthritis [2,6,11].

1.2.2 Priming

It is essential that neutrophils only respond appropriately to inflammatory stimuli, and do not release their cytotoxic products to cause tissue damage. To prevent inappropriate activation, neutrophils undergo a two-step activation process, initially being primed into a state of heightened responsiveness, before becoming fully activated when they encounter a stimulus [3]. Neutrophils circulate in the body in an inactive state, but can be primed by a variety of stimuli including bacterial peptides, and cytokines such as GM-CSF. The surface expression of a number of plasma membrane receptors is increased (either in number or by a change in affinity) when neutrophils are primed, and the primed neutrophils exhibit enhanced degranulation, phagocytosis and production of ROS in response to agonists. Thus, priming greatly enhances bacterial killing [3,47]. These changes occur rapidly, and the speed of response is facilitated by the storage of pre-formed receptors and subunits, that can be rapidly mobilised to the cell surface independently of *de novo* protein synthesis. Priming does, however, also induce longer-term effects that are dependent on *de novo* protein biosynthesis. These products of *de novo* biosynthesis include proteins that extend the functional life-span of the neutrophil and also a wide range of cytokines that act as further mediators of the immune response [17,48].

1.2.3 Adhesion, transmigration and chemotaxis

For neutrophils to respond to infection they must reach the site of the immune challenge, which requires them to leave the circulation and enter the tissue. This process involves adhesion to, and transmigration through the blood vessel wall, which is mediated by both neutrophil and endothelial cell membrane receptors, the expression of which can be differentially regulated by cytokines [49–51]. The adhesion receptors can be grouped into 3 types that act sequentially; selectins, integrins and immunoglobulin receptors. The selectins are responsible for the initial tethering of the neutrophil to the endothelium and its subsequent rolling along the vessel wall. L-selectin is the major neutrophil selectin and is constitutively expressed, whereas P- and E-selectin are expressed on endothelial cells [51,52]. Transient binding of these receptors to their ligands facilitates rolling of the neutrophil onto the endothelium. Following attachment, L-selectin is shed and then expression of neutrophil integrins is stimulated by cytokines and factors released from endothelial cells [51,52]. Rolling arrest and firm adhesion of the neutrophil to the endothelium is mediated by the neutrophil integrins that interact with the endothelial intercellular adhesion molecule (ICAM) receptors ICAM-1 and ICAM-2, ICAM-1 expression is upregulated by cytokines. Integrin CD11b/CD18 (Mac-1 or CR3) and endothelial ICAM-1 cooperate to enable neutrophil crawling to a suitable site for neutrophil transmigration where the neutrophil docks [51–53]. At this point, docking of the neutrophil is thought to lead to changes in the

endothelial cell morphology to aid neutrophil transmigration between cells, in a process known as diapedesis [52,54]. Paracellular diapedesis is mediated by molecules redistributed to endothelial cell junctions, including the immunoglobulins PECAM-1, ICAM-1, ICAM-2 and JAM-A, -B and –C [51,52]. Different molecules mediate transmigration in a stimulus dependent manner, for example ICAM-2, PECAM-1 and JAM-A mediate neutrophil transmigration in response to IL-1β but not TNFα [55]. In contrast to this paracellular transmigration of neutrophils through endothelial cell junctions, a minority of emigrating neutrophils undergo transcellular migration, usually under conditions of very high ICAM1 expression. During transcellular migration, neutrophils migrate through small continuous endothelial membrane-associated channels called vesiculo-vacuolar organelles. This process is mediated by many of the same paracellular migration facilitating molecules [49,56,57].

Once neutrophils enter the tissue they continue to move towards the site of infection, following a gradient of increasing concentration of chemoattractants such as bacterial peptides, complement fragments and cytokines including IL-8 and TNFα [58]. Neutrophils exposed to these agents change shape and exhibit polarity, with a leading edge and trailing end. Migration occurs via extension of actin-rich protrusions, known as pseudopodia, from the leading edge of the neutrophil, and contraction of the trailing end [58]. During this process, chemokine receptors cluster at the

leading edge of the neutrophil to facilitate detection of the chemotatic agents [59].

1.2.4 Pathogen recognition and phagocytosis

By virtue of various plasma membrane receptors, neutrophils recognise pathogens and other particles that have been opsonised by immunoglobulins (Ig) and/or complement fragments. The number and affinity of these receptors is increased when neutrophils are primed by cytokines [3,48]. One group of receptors recognises and binds the bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP); fMLP is a potent chemoattractant and activator of neutrophils and fMLP receptors bind this molecule and its analogues. Complement receptors are also upregulated during priming; the soluble complement factors C3a and C5a initiate neutrophil chemotaxis and secretion, whereas surface-bound fragments of C3 coat and opsonise pathogens and stimulate neutrophil adhesion and phagocytosis via their receptors CR1 and CR3 (CD11b/CD18 or Mac-1) [60,61]. Particles are also opsonised by being coated with immunoglobulins to further enhance recognition by neutrophils. Neutrophils express a family of immunoglobulin receptors that recognise the Fc domain of IgG, called Fcγ receptors. The immunoglobulin receptors FcγRII (CD32) and FcγRIII (CD16) are constitutively expressed on the surface of neutrophils, whereas FcγRI (CD64) is absent on resting neutrophils, but, its surface expression is induced by cytokines, such as

interferon-γ (IFNγ) and GM-CSF [62]. FcγRI binds monomeric IgG with high-affinity, FcγRII will also bind monomeric IgG with low affinity but it has a greater affinity for dimers or aggregates of IgG. There are 2 isoforms of FcγRIII, FcγRIIIa and -b, but only FcγRIIIb (CD16b) is expressed on neutrophils. FcγRIIIb is constitutively expressed at a higher concentration than the other Fcγ receptors on the neutrophil surface (100,000–200,000 molecules per cell). FcγRIIIb is anchored to the membrane via a glycosyl-phosphatidylinositol (GPI) linkage which enables cleavage of this receptor from the cell surface [1,63]. Shedding, via GPI-cleavage, alongside mobilisation of internal stores of this receptor, allows dynamic regulation of expression on the neutrophil surface. Stimulation with cytokines such as GM-CSF leads to rapid shedding and concurrent replenishment of FcγRIIIb on the neutrophil surface [64].

Neutrophils also express Toll-like receptors (TLR) that recognise structurally-conserved pathogen-associated molecular patterns (PAMPs) [65]. TLRs are part of the IL-1/TLR superfamily and neutrophils express all 10 TLRs (TLR1-10) except TLR3. Expression can be upregulated by priming; for example GM-CSF increases expression of TLR2 and TLR9 [66]. This facilitates recognition of a variety of bacterial products including lipoproteins, lipopolysaccharide (LPS), peptidoglycans, flagellins and RNA (which facilitates recognition of viruses) [67]. TLR agonists activate MAPK, and subsequently NF-κB signalling pathways, resulting in

initiation of phagocytosis [68]. TLR receptor engagement also leads to increased degranulation, production of ROS and cytokine release [65,66,69].

Once a neutrophil recognises a pathogen by virtue of these receptors, it engulfs it by phagocytosis [70]. There are two major receptor-dependent mechanisms by which this occurs; particles coated by opsonins bind to complement receptors on the surface of the neutrophil and rapidly sink into the cell without stimulating significant surface protrusions [71,72]. Alternatively, activation of immunoglobulin receptors by IgG coated particles drives actin polymerisation and the formation of protruding pseudopodia that extend around the pathogen and fuse to engulf it. The resulting membrane bound vesicle is drawn into the cell and is termed the phagosome. This process in disrupted by addition of cytochalasin B *in vitro* [71,72]. Neutrophil granule proteins and NADPH oxidase activity during the respiratory burst then act in concert to create an intra-phagosomal environment that can destroy the engulfed pathogen [44].

1.2.5 Degranulation

Neutrophil granules contain a vast array of antimicrobial substances that are delivered to the phagosome during degranulation. However, these can also be delivered to the cell surface to facilitate secretion of the molecules [73]. There are a number of subsets of granules, distinguishable by their protein content. All subsets share common structural features, such as a phospholipid bilayer membrane and an intragranular matrix containing

the granule proteins. It is thought that the protein contents of each granule type is determined by the timing of development during neutrophil maturation [74,75]. The types of granules are broadly grouped into peroxidase-positive (also known as the azurophilic or primary granules) and peroxidase-negative, with the peroxidase-negative group further subdivided into the specific (secondary), gelatinase (tertiary) and the secretory vesicles [1]. These granules contain anti-microbial proteins and stores of pre-formed receptors; the contents of each type is summarised in Table 1.1. The defining protein of the peroxidase positive group is myeloperoxidase (MPO). This heme dependent protein oxidises chloride (and other halides) and reacts with hydrogen peroxide (H_2O_2) formed by the NADPH oxidase to produce hypochlorous acid (HOCl) [76]. This process is discussed in more detail in section 1.2.6. Another major component of these primary granules are the α-defensins, which are antimicrobial peptides, and are estimated to constitute 5% of the total neutrophil protein content [77]. Generally, the larger secondary granules are richer in antimicrobial proteins than the smaller tertiary granules; these antimicrobial products are mobilised to the phagosome or released extracellularly and are crucial for killing [73]. The tertiary granules contain a number of matrix-degrading enzymes and pre-formed receptors and are more easily exocytosed to provide these products to the cell surface [78].

Peroxidase	Granule	Contents
Positive	Azurophillic/Primary	Myeloperoxidase Defensins BPI Cathepsin G Elastase Proteinase 3 Azurocidin Lysozyme Alkaline phosphatise Hydrolases Phospholipase A_2, C and D
Negative	Specific/Secondary	Lysozyme Lactoferrin fMLP receptor MAC-1 (CD11b/CD18) Laminin receptor Cytochrome b_{558} Gelatinase Collagenase
Negative	Gelatinase/Tertiary	fMLP receptor MAC-1 (CD11b/CD18) CD45 receptor Laminin receptor Cytochrome b_{558} B2 microglobulin Gelatinase Diamine oxidase H+-ATPase Tetranectin DAG lipase
Negative	Secretory vesicles	fMLP receptor MAC-1 (CD11b/CD18) LFA-1 (CD11a/CC18) Complement receptor-1 Cytochrome b_{558}

TABLE 1.1: Protein contents of neutrophil granules [1]

The secretory vesicles have the highest propensity for extracellular release, and contain a variety of membrane-associated receptors, such as CD11b/CD18 and other complement receptors, which are mobilised to the cell surface early in the inflammatory response [78–80].

In vitro addition of nanomolar concentrations of agonists such as fMLP can stimulate mobilisation of secretory vesicles without significantly affecting the other granules [80]. Thus, the granule contents of neutrophils provide components for various stages of neutrophil activity, with the anti-microbial peptides of the primary and secondary granules acting alongside the oxygen-dependent activity of the NADPH oxidase complex to orchestrate bacterial killing [44].

1.2.6 Production of ROS by neutrophils

Neutrophils produce antimicrobial oxidants that are released into the phagosome or extracellularly and act in concert with the granular antimicrobial peptides during microbial killing. Reactive oxygen species (ROS) are produced by the reduction of molecular O_2 by the multi-component NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase in a process known as the respiratory burst. The primary product of the NADPH oxidase (O_2^-) is relatively inactive but it is unstable and it rapidly dismutates into hydrogen peroxide (H_2O_2). Under certain conditions and in the presence of metal salts, these ROS can also be converted into the highly reactive hydroxyl free radical (·OH)

[44]. H_2O_2 is itself microbicidal at high concentrations but the granule enzyme myeloperoxidase can further convert it into hypochlorous acid (HOCl), which is a much more potent microbicidal agent [81]. This process is summarised in Figure 1.2. The NADPH oxidase uses the reducing power of NADPH to reduce molecular oxygen, resulting in $NADP^+$, which is subsequently converted back to NADPH via the hexose monophosphate (HMP) shunt. The HMP shunt, also known as the pentose phosphate pathway, consists of two distinct phases; NADP becomes reduced to NADPH during the first (oxidative) phase, when glucose-6-phosphate (G6P) is converted into 6-phosphogluconolactone (6PGDL) by G6P dehydrogenase (G6PDH). The action of G6PDH is allosterically stimulated by NADP. This is followed by the non-oxidative phase during which 6PGDL is decarboxylated to produce the 5-carbon sugar ribulose-5-phosphate [82]. In this way neutrophils can regenerate NADPH to provide a source of reducing power for the oxidase; however, resting neutrophil pools of NADP/H are insufficient to fuel the oxidase, and are increased greater than 3-fold upon neutrophil activation with fMLP or phorbyl myristate acetate (PMA), requiring new input of phosphorylated NAD [83].

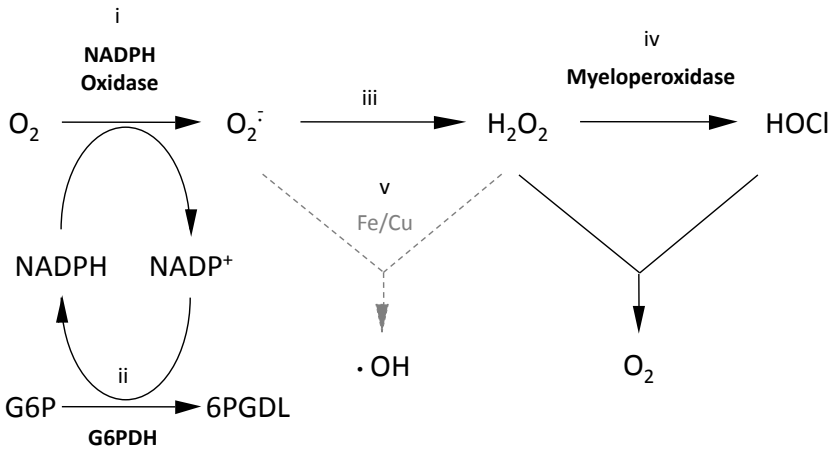

FIGURE 1.2: ROS production in neutrophils. A. i. The NADPH oxidase complex uses the reducing power of NADPH to reduce molecular oxygen, forming O_2^-. **ii.** Oxidised $NADP^+$ is converted back to NADPH during the hexose monophosphate (HMP) shunt **iii.** O_2^- is relatively unstable and spontaneously forms secondary oxidants. **iv.** Neutrophils contain myeloperoxidase which catalyses production of HOCl. **v.** Under certain conditions and in the presence of transition metal salts, O_2^- and H_2O_2 may react to form the highly reactive ·OH radical (adapted from [84]).

The core NADPH oxidase consists of the membrane bound $p22^{PHOX}$ (CYBA) and $gp91^{PHOX}$ (CYBB) subunits that form cytochrome b_{558}, and the cytosolic components p40 ^{PHOX} (NCF4), p47 ^{PHOX} (NCF1), p67 ^{PHOX} (NCF2), and is associated with Rac1 or Rac2 which are low-molecular weight G proteins of the Rho guanosine triphosphatase (GTPase) family [85]. The Rac proteins are held in an inactive state by the Rho guanine nucleotide dissociation inhibitor (RhoGDI) which binds and prevents their association at the membrane [86]. Upon neutrophil priming, the cytosolic components of the oxidase migrate to the membrane to enable assembly of the enzyme, that allows a rapid response if the cell is further

activated, in a process regulated by phosphorylation [87]. gp91^{PHOX} binds the electron carrying components of the oxidase including the flavin adenine dinucleotides (FAD) and two heme molecules, which are required for electron transfer from NADPH to molecular oxygen [88]. p22^{PHOX} is the second subunit of the heterodimeric flavocytochrome and is associated with gp91^{PHOX} at the membrane. p22^{PHOX} extends a tail into the cytoplasm and is bound by p47^{PHOX}; this adaptor peptide is phosphorylated upon activation and is responsible for bringing the cytosolic subunit of the oxidase to the membrane bound catalytic core [43]. This facilitates the association of gp91^{PHOX} with p67^{PHOX} which is thought to catalyse the electron transfer to molecular oxygen. p67^{PHOX} also binds the Rac protein, which is crucial for oxidase assembly [89] (Fig. 1.3). The function of the final oxidase component p40^{PHOX} remains unclear; p40^{PHOX} associates with p67^{PHOX} and p47^{PHOX} in the cytosol and it has been suggested that it is involved in the regulation of p67^{PHOX} action due to their tight association. However, this is yet to be confirmed and evidence for both positive [90–92] and negative [93,94] regulatory roles has been presented.

Much of what is known about the function of the oxidase has come from the study of the phagocytic cells of patients with chronic granulomatous disease (CGD), which is characterised by a variety of genetic defects in the oxidase genes [95,96], however, no CGD patients have been identified

with mutations of $p40^{PHOX}$ [43]. Generally, CGD leads to the inability to produce superoxide and is often characterised by severe infections [97–99]. The incidence of infection, however, does differ between patients, and some have been documented as having relatively few bacterial infections, despite being unable to produce substantial levels of phagosomal oxidants, and relying solely on O_2 independent microbicidal activities [100].

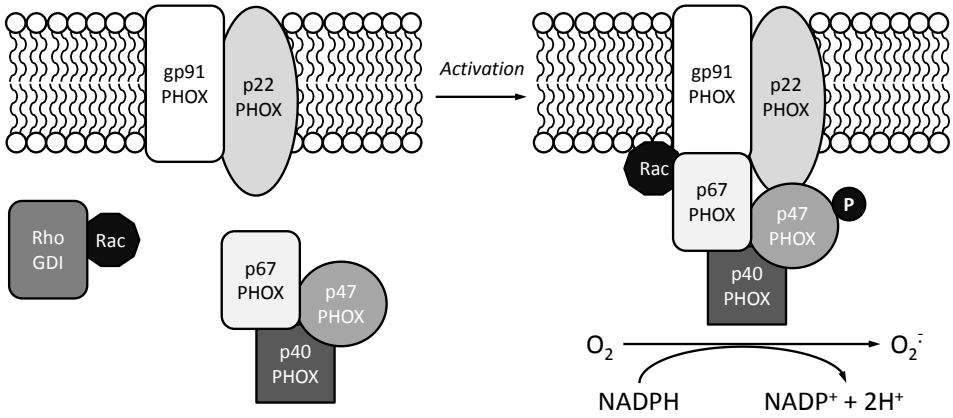

FIGURE 1.3: Assembly of the multi-component NADPH oxidase upon neutrophil activation. In resting neutrophils the membrane and cytosolic subunits of the NADPH oxidase are not associated with one another, and the Rac protein is held in the cytosol by the Rho guanine nucleotide dissociation inhibitor (RhoGDI). Upon neutrophil activation, various signalling pathways trigger release of the adaptor protein Rac and phosphorylation of the $p47^{PHOX}$ subunit, leading to migration of the cytosolic subunit to the membrane and its association with the flavocytochrome to form the functional oxidase. The oxidase complex then catalyses reduction of molecular oxygen using NADPH as a co-factor.

The neutrophil respiratory burst coincides with degranulation; the oxidant species produced during the neutrophil respiratory burst have microbicidal activity, as free radicals readily react with organic

molecules, and the catalysis of H_2O_2 to HOCl by the granule protein myleoperoxidase is one example of the coordinated nature of the neutrophil killing mechanisms [44]. However, production of ROS also changes the conditions within the phagosome to enhance other killing mechanisms; the high ROS concentration results in an accumulation of anionic charge, and the pH within the phagosome transiently rises from pH 6 to around pH 7.8, triggering an influx of potassium ions into the phagosome across the membrane. This increase in ionic strength leads to activation of cationic granule proteins, including elastase and cathepsin G, that act to destroy the bacteria [101]. Thus, the concerted action of the two neutrophil killing mechanisms is important for effective bacterial killing.

1.3 Neutrophil Apoptosis

It is vitally important that host cells and tissues are protected from the cytotoxic molecules that are used by neutrophils to kill pathogens, as they have the capacity to cause bystander tissue injury. This is achieved in part by the two-stage activation of neutrophils, but it is complemented by the tight regulation of neutrophil apoptosis. Neutrophils have a short half-life in the circulation and in the absence of pro-inflammatory signals will undergo apoptosis [38]. This constitutive apoptosis is transiently delayed during immune challenge to allow neutrophils to carry out their anti-microbial activities. Controlled neutrophil apoptosis, however, is also

crucial for the resolution of inflammation following pathogen killing. If neutrophils did not undergo controlled death in this manner, uncontrolled death and secondary necrosis would lead to the release of intracellular cytotoxic proteases and exacerbate the acute inflammatory state [45,102]. Neutrophil apoptosis is triggered either by the withdrawal of positive survival signals or by exposure to negative death signals, which respectively trigger the intrinsic and extrinsic apoptotic pathways. Morphologically, apoptotic neutrophils are distinct from their healthy counterparts. Apoptosis results in condensation of the nuclear chromatin, nuclear fragmentation, and degradation of DNA and proteins, leading to condensation of the nucleus which loses its characteristic multi-lobed shape and becomes more rounded, disappearance of the granules and overall cell shrinkage [42]. Once neutrophils have undergone apoptosis they are cleared by other phagocytic immune cells, such as macrophages to prevent release of proteases [46].

Neutrophils may be exposed to multiple mediators of inflammation, so their fate depends on the balance between pro-survival and pro-apoptotic cues. Cell death is facilitated by activation of a cascade of caspase (cysteine-aspartic protease) cleavage. Caspases exist in inactive, pro-forms in the cell until, following an apoptotic signal, they are sequentially cleaved and activated in a cascade mechanism. This cascade culminates in the cleavage and degradation of a number of proteins in the cell and

ultimately cellular disassembly and death. Caspases are activated via both intrinsic and extrinsic apoptotic signalling, and it is reported that their function can be further mediated by ROS [103,104].

1.3.1 The intrinsic apoptotic pathway

Constitutive neutrophil apoptosis in the absence of inflammatory signals is achieved by constitutive expression of pro-apoptotic members of the Bcl-2 family. These proteins, including Bax, Bad, Bak and Bid, have a relatively long half-life and their expression is not greatly affected by agents that induce or delay apoptosis [5,105]. Thus, dynamic regulation of neutrophil apoptosis is mainly achieved by the anti-apoptotic Bcl-2 family members. The anti-apoptotic Bcl-2 family member Mcl-1 plays a key role in neutrophil apoptosis. There is some evidence that neutrophils also express the other anti-apoptotic Bcl-2 family members A1 (Bfl-1), and possibly to a lesser extent Bcl-X_L, but not Bcl-2 [105,106]. Mcl-1 is responsive to stimulation by pro-apoptotic stimuli such as cytokines, and as such is the key protein responsible for the dynamic regulation of neutrophil apoptosis [38,105]. Mcl-1 is a short lived protein and its rates of transcription and protein turnover control the rate of neutrophil apoptosis. Pro-inflammatory cytokines, such as GM-CSF and other inflammatory indicators such as hypoxia, can stimulate transcription of Mcl-1 in neutrophils, although this increase in transcription is modest compared to the change in protein levels, suggesting that this may not be the major way cellular levels are

controlled [105,107,108]. Post-transcripitonal splicing can also modulate the protein levels, as a short version of the transcript is generated when exon 2 is removed, forming a protein with pro-apoptotic activity that inhibits the action of full-length Mcl-1 [109,110]. The major way in which levels of Mcl-1 are controlled in neutrophils is via post-translational modifications. Mcl-1 has a number of phosphorylation sites and its phosphorylation at different sites can have a differing effect on its stability; for example phosphorylation by ERK stabilises Mcl-1 [111], whereas phosphorylation at alternative sites by Glycogen synthase kinase-3 (GSK3), results in its destabilisation [112,113], and hyper-phosphorylation of Mcl-1 targets it for degradation by the proteosome [114]. Because of these differing outcomes of phosphorylation on its stability, Mcl-1 has been described as the factor that integrates the opposing actions of pro-survival and pro-apoptotic signalling pathways [115].

These biological properties of Mcl-1 make it suited for the acute and dynamic regulation of apoptosis required for neutrophils; in the absence of continued expression, Mcl-1 is rapidly broken down and neutrophil apoptosis is triggered. The sensitivity of Mcl-1 to pro-inflammatory mediators however, means that its expression can be increased and the protein can be stabilised, extending neutrophil life-span to facilitate bacterial killing. The naturally short-life span of the protein encourages

neutrophil apoptosis, facilitating resolution of inflammation following removal of inflammatory stimuli [38,102,116].

The pro-apoptotic Bcl-2 family members mediate apoptosis via the intrinsic or mitochondrial pathway by controlling permeabilisation of the outer mitochondrial membrane. Assisted by other pro-apoptotic proteins such as Bid, Bak and Bax oligomerise and promote formation of pores in the mitochondrial outer membrane. This disruption of the membrane leads to release of a number of pro-apoptotic factors including cytochrome c. Cytochrome c along with its interacting protein, apoptotic protease activating factor 1 (APAF1), recruits and activates caspase 9, which goes on to activate the downstream effector caspase, caspase 3, culminating in cell death [4,5,117]. Anti-apoptotic Mcl-1 contains three BH3 (Bcl-2 homology domains) that allow it to associate with other Bcl-2 proteins [115,118]. In this way, Mcl-1 can neutralise the pro-apoptotic activity of Bcl-2 family proteins, by sequestering them and inhibiting activation of pore-formation at the mitochondrial membrane [38,119,120]. Under conditions of stress, the Mcl-1 antagonist NOXA, causes release of the sequestered pro-apoptotic Bcl-2 proteins and apoptosis is triggered [121–123].

1.3.2 The extrinsic apoptotic pathway

Neutrophil apoptosis can also be triggered extrinsically, by engagement of death receptors on the neutrophil surface. Engagement of a death receptor leads to the activation of the initiator caspases (including caspases 8, 9, 10

and 12), which go on to activate the downstream effector caspases (including caspases 3, 6 and 7) [124]. These extrinsically-activated apoptotic signalling pathways usually bypass the intrinsic or mitochondrial apoptotic pathway, and as such are not regulated by the Bcl-2 family. However, the pro-apoptotic Bcl-2 family member Bid can be activated by caspase-8, suggesting there is some level of crosstalk between the two pathways [124,125]. The death receptors are generally transmembrane proteins containing an intracellular motif known as a death domain. Upon receptor engagement these death domains translate this signal to the intracellular apoptotic machinery by association with a variety of apoptotic factors [126,127]. Two of the most well characterised neutrophil death receptors are TNFR1 and Fas (also known as TNFR superfamily member 6), engagement of which activates intracellular caspase cleavage cascades via initial activation of caspase 8 in a similar manner for both receptors [127]. Fas ligand (FasL) activates the Fas receptor, and TNFα is the receptor for the TNFR group, and in both cases binding of the trimeric ligand leads to trimerisation and cross-linking of receptors. This results in clustering of the intracellular death domains to initiate recruitment of apoptotic adaptor proteins; either the Fas-associated death domain-containing protein (FADD) or TNFR-associated death domain-containing proteins (TRADD). These molecules also contain death effector domains that facilitate interaction with apoptotic molecules. FADD associates with the pro-form of the initiator caspase, caspase 8 and induces autoprocessing to the active

form [128]. This complex of Fas, FADD and pro-caspase 8 has been termed the death-inducing signalling complex (DISC) [124,129]. TRADD can also associate with FADD to initiate apoptosis in this manner [130], but TRADD alternatively associates with a variety of other secondary adaptor molecules that can lead to activation of transcription factors and production of pro-survival signals [131,132]. At low concentrations, TNFα specifically induces expression of the pro-survival protein A1 (Bfl-1) in neutrophils. Thus, TNFα has opposing concentration dependent effects on neutrophil apoptosis [133].

1.4 Neutrophils in chronic inflammation

Neutrophils are critically important in host defence, but due to their cytotoxic contents, they have great capacity to cause tissue damage if their function and apoptosis are not tightly regulated. The potential of neutrophils to exacerbate chronic inflammation is now appreciated, and they have been shown to contribute to rheumatoid arthritis (RA), chronic obstructive pulmonary disease (COPD) and Behçet's disease [2,6]. Neutrophils have been shown to be inappropriately activated in these conditions, leading to the release of cytotoxic products, and also cytokines and chemoattractants [2]. One inflammatory disease in which the contribution of neutrophils to the inflammatory state is of great interest, is RA [2,6,11]. During active disease, the normally acellular joint becomes infiltrated with immune cells, of which neutrophils are by far the most

numerous accounting for up to 90% of the cells in the synovial fluid of a typical rheumatoid knee joint [7,8]. The etiology of RA remains incompletely understood, but neutrophils are thought to contribute to the characteristic inflammation and tissue damage observed in the rheumatoid joint [2,6,11].

1.4.1 Rheumatoid arthritis

RA is a systemic, autoimmune inflammatory disease, mainly characterised by symmetrical polyarthritis of the diarthrodial (free moving) joints, especially the small joints of the hands and feet [14,16]. The onset of the disease is subtle, but the predominant symptoms of established disease are pain, swelling and stiffness of the peripheral joints. The symptoms can be mild and self-limiting in some patients, whereas in others they can rapidly lead to severe deformity and loss of function. Further complications of systemic inflammation include an increased risk of vasculitis, respiratory and cardiac diseases, which can lead to profound morbidity and reduced life expectancy [14,134,135]. Inflammation of the synovium (synovitis) is fundamental to the pathophysiology of RA, and correlates with disease progression; characteristically a pannus of proliferative and invasive synovial tissue forms locally and exacerbates joint erosion [14,136]. Joint destruction resulting from this persistent synovitis is typically rapid, and is detectable by radiographic techniques within the first 2 years of onset of disease in more than 70% of patients. This predates functional disability by a number of years and often once physical deformity is presented,

irreversible joint damage has already occurred [14,136]. Because of this, early and effective drug intervention is imperative in RA to prevent accumulation of irreversible joint damage.

1.4.2 Clinical measurements and treatment of RA

A classification system for diagnosis of RA was developed by the American College of Rheumatology. It describes defining criteria of the disease, including corporal features evident upon examination, such as morning stiffness and swelling of the joints, and those that require further investigation, such as radiographic erosions and the presence of inflammatory serum markers [137]. A number of biochemical markers have been identified as being of prognostic value in RA; the erythrocyte sedimentation rate (ESR) and serum C-reactive protein (CRP) are inflammatory markers associated with RA [138,139]. However, joint damage has been shown to progress in the absence of these inflammatory markers [16]. More recently, serum levels of the autoantibodies rheumatoid factor (RF), and anti-cyclic citrullinated peptide antibodies (anti-CCP) have been associated with more severe joint damage, and anti-CCP is reported to be more specific for RA [16,140–143]. However, RA is a very heterogeneous disease and the presence or absence of these markers does not always correlate with disease activity or progression, making a multifaceted diagnosis of prime importance [16].

Typically, treatment for RA includes a combination of non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs) and the newer class of biological-response modifiers (biologics) that target specific cytokine mediators of disease [144–147]. Analgesic NSAIDs improve the symptoms of disease, although they do little to alter its progression; they generally act by inhibiting cyclooxygenase and decreasing production of inflammatory prostaglandins [145,148]. As their name suggests, DMARDs modify disease, slowing clinical and radiographic progression. They are thought to act by either directly or indirectly affecting inflammatory pathways mediated by cytokines [144,148]. DMARDs, including methotrexate, hydrochloroquine and sulfasalazine, are now used as the first-line therapy for all newly diagnosed cases of RA [149]. Many patients will remain well-controlled through a combination of DMARD therapy and NSAIDs for management of pain and inflammation. However, those with more severe disease may be given biologics that selectively inhibit specific molecules of the immune system. These include inhibitors of cytokines, such as: etanercept, infliximab and adalimumab, that target TNFα; anakinra that targets interleukin-1; tocilizumab which targets the IL-6 receptor; rituximab an anti-CD20 antibody that targets CD20 positive cells, specifically B-cells; and abatacept, a fusion protein that inhibits the co-stimulation of T-cells [144,146,149]. These therapies are very effective in the majority of patients but a significant cohort remain refractory to treatment or develop resistance [15,150]. Due to the progressive

nature of the disease, bringing it under control quickly is vitally important to prevent further irreversible joint damage. Hence, research into alternative treatment strategies and diagnostic markers of response in RA is ongoing.

A system has been developed for scoring the severity of disease in RA, known as the disease activity score or DAS. This combines measurement of the ESR, the number of tender and swollen joints and a self-measurement of pain, into a numerical value. The classification of the scoring system is shown in Table 1.2. Generally, patients are only prescribed biologic therapies for RA if they sustain a high DAS following DMARD therapy. A patient is considered to respond to therapy if they show an improvement in their DAS of ≥1.2 [151].

DAS	Classification
≤ 2.6	Remission
2.7 - 3.2	Low
3.3 - 5.0	Moderate
≥ 5.1	High

TABLE 1.2 DAS score classification system [151].

1.4.3 The immune system in RA

The etiology of RA remains incompletely understood. However, it is thought that genetic factors make individuals more susceptible to producing auto-antibodies in response to environmental triggers, such as

cigarette smoke. Autoimmunity is thought to be established a number of years before the onset of symptoms [152]. In active disease, a number of immune cell types of both the adaptive and innate immune systems infiltrate the normally acellular joint. Immune cells such as lymphocytes, macrophages and neutrophils infiltrate the synovial lining of the joint and the proliferative pannus, comprised of activated synovial fibroblasts [153,154]. Here, the pro-inflammatory mediators secreted by these cell types activate osteoclasts at the pannus/cartilage junction, and these cells, together with neutrophils and macrophages produce proteases. This is the site of active cartilage and bone destruction, and the perpetual recruitment and activation of cells, involving many elements of the immune response, alongside the production of cytotoxic products leads to the irreversible damage observed in the rheumatoid joint [8,10,155].

1.4.4 The neutrophil in RA

Neutrophils account for up to 90% of the cells in the synovial fluid of a typical rheumatoid knee joint [7,8] and have been observed at the pannus/cartilage junction [8,10]. A number of animal models of inflammatory arthritis also show that *in vivo* neutrophils are the first immune cell types to infiltrate the joint and correlate with early measures of inflammation in these models [156]. The cytokines inappropriately produced during chronic inflammation lead to priming of circulating neutrophils and their chemotaxis towards the rheumatoid joint, which contains high levels of

pro-inflammatory cytokines such as IL-1β, IL-6, GM-CSF and TNFα [157]. Neutrophils aspirated from rheumatoid joints display an activated phenotype such as: increased mRNA expression, for transcripts such as those for chemokines, cytokines and destructive proteins such as matrix-metalloproteinases (MMP); increased ROS production; and increased surface expression of markers such as class II major histocompatibility complex (MHCII) molecules, and the IgG receptor FcγRI (CD64) [17,158–160]. Neutrophil apoptosis is also delayed for several days when they enter the inflammatory environment of the rheumatoid joint [12,13]. These primed neutrophils, that are present in large numbers, can be activated by molecules found within the rheumatoid joint, such as IgG-containing immune complexes. Immune complexes in RA are aggregates of autoantibodies and antigens that interact with neutrophils via the Fcγ receptors. They are the major neutrophil activating factors in the rheumatoid joint. Large insoluble immune complexes interact via FcγRII and are usually phagocytosed by neutrophils with few side effects; however, smaller, soluble immune complexes interact via both FcγRIIIb and FcγRII, and stimulate secretion of destructive ROS and granule enzymes from primed neutrophils into the extracellular milieu [161–163]. Indeed, comparison of neutrophils from the synovial fluid to those in the peripheral blood, suggest that those in the joint had undergone degranulation, as they had lower levels of intracellular granule enzymes [164]. Immune complexes can also be deposited onto tissue surfaces, such as the

pannus, which can stimulate a process known as frustrated phagocytosis. During this, the neutrophil releases its cytotoxic products directly at the pannus surface, leading to a high localised concentration of damaging agents linked to cartilage destruction [11]. Additionally, increased release of ROS from neutrophils is thought to be responsible for oxidative stress in the tissue, another hallmark of RA [160]. Immune complexes and cytokines in the joint can also induce expression of further cytokines (such as IL-1β and TNFα) and chemoattractants (such as LTB_4 and IL-8) from neutrophils, recruiting and activating further immune cells and contributing to the perpetuation of inflammation in the joint [17].

Taken together, the infiltration of large numbers of activated neutrophils into the rheumatoid joint, and the presence of their pro-inflammatory products in the extracellular milieu, strongly indicates a role for these cells in the pathophysiology of RA. If their function and/or apoptosis are not appropriately controlled, these cells have great capacity to cause damage in inflamed tissue, due to the release of tissue-damaging proteases, ROS and cytokines. As such, neutrophils represent an attractive target in RA, a disease in which it is of interest to identify novel molecular targets due the high degree of heterogeneity in both the disease and the response to treatment. A recent high-level screen of genes expressed by neutrophils in a number of inflammatory diseases (RA, Beçet's disease and scleroderma), was carried out (prior to the start of this PhD) with the aim of identifying

neutrophil biomarkers of inflammatory disease. A major finding was a remarkably high level of transcripts for the NAMPT gene observed in all samples (Edwards *et al.* unpublished data). This gene is of particular interest due to its recently defined role in the regulation of neutrophil apoptosis [19] and its correlation with inflammatory disease [18,165].

1.5 NAMPT

NAMPT is a highly conserved and ubiquitously-expressed protein with a number of described functions. NAMPT is a nicotinamide phosphoribosyltransferase enzyme which catalyses a key stage in the mammalian NAD salvage pathway, but it has also been described as having a signalling function as a cytokine when released from immune cells: somewhat controversially, it has also been described as an adipokine released from adipose tissue [23,166]. These multiple functions of NAMPT have led to the use of other names for this protein, such as pre-B-cell colony stimulating factor (PBEF) and visfatin, the derivation of which are described below. The term NAMPT, has now been approved by the Human Genome Organisation Gene Nomenclature Committee. The following section covers the research history of NAMPT, the discoveries of the various proposed functions of this protein, and also NAMPT gene and protein structure.

1.5.1 NAMPT history

Human NAMPT was first identified and cloned from activated human peripheral blood lymphocytes by Samal and colleagues in 1994 [167]. With the aim of identifying new factors involved in early B-cell development, Samal *et al*. screened an activated human peripheral blood lymphocyte cDNA library with a degenerate oligonucleotide probe, designed on the basis of the similarity of the signal peptidase processing site coding sequences of cytokines such as GM-CSF, IL-1β, IL-2, IL-3 and IL-6. They identified a novel molecule which functioned to synergise the pre-B-cell colony forming activity of stem cell factor and IL-7, and designated it pre-B-cell colony stimulating factor (PBEF). It was defined as a novel cytokine that acts on early B-lineage precursor cells. PBEF lacked the characteristic signal peptide necessary for extracellular secretion, but more characteristically of a cytokine, it contained multiple TATT motifs in the 3' untranslated region and was found to be upregulated in response to pokeweed mitogen, cycloheximide and PMA in activated lymphocytes and COS cells [167].

It was not until 2001 that another function of PBEF/NAMPT was identified from an unrelated field. Martin *et al*. [168] identified that a gene they cloned from *Haemophilus ducreyi* showed significant sequence homology to the mammalian PBEF cloned by Samal *et al*. [167]. Members of the bacterial family *Pasteurellaceae* can be distinguished, in part, by

their ability to synthesise NAD from nicotinamide, known as V-factor independence. V-factor dependent strains require NAD supplemented media for growth in the laboratory. Martin and colleagues [168] cloned the gene responsible for this phenotype from *H. ducreyi* and named it *nadV*. They demonstrated that when transformed into other V-factor dependent species of bacteria, *nadV* conferred NAD independence. Sequence analysis of the *nadV* gene then surprisingly revealed significant homology to mammalian PBEF [168]. Shortly after this, Rongvaux *et al*. [30] demonstrated that mammalian PBEF also had nicotinamide phosphoribosyltransferase activity and was able to confer V-factor independent growth to a V-factor dependent strain of bacteria, confirming the high degree of evolutionary conservation of this gene. NAMPT (nicotinamide (NAM) phosphoribosyltransferase (PT)) has since been approved as the official name for this gene. This activity of NAMPT was corroborated by Revollo *et al*. in 2004 [169] who reconstituted the mammalian NAD biosynthesis pathway *in vitro* using purified recombinant NAMPT and NMNAT (nicotinamide mononucleotide adenylyl transferase) [169].

In 2005 a further function of NAMPT was proposed when Fukuhara *et al*. [31] identified NAMPT as a novel visceral fat-derived hormone which they termed visfatin. They showed that visfatin was highly enriched in visceral fat as compared to subcutaneous fat, and that plasma visfatin was

elevated in obesity. They also suggested that visfatin exerted insulin mimetic effects on its target cells, such as enhanced glucose uptake and increased triglyceride synthesis, and that in mice it lowered plasma glucose by binding to and activating the insulin receptor at a site distinct from insulin. However, subsequent studies have yielded conflicting results with regard to the relationship between circulating visfatin, obesity and insulin resistance and none have been able to replicate the insulin mimetic effects of visfatin [26,170–172]. It was later demonstrated that differences in the detection of visfatin by different immunoassays may be responsible for the conflicting observations [173]. The Fukuhara *et al*. paper was eventually retracted in 2007, and it remains unclear whether visfatin/NAMPT is a true adipokine and how it is related to insulin sensitivity.

1.5.2 Current opinion on NAMPT function

It has been robustly demonstrated, via biochemical and structural analyses, that NAMPT functions in NAD biosynthesis [27,29,168,174,175]. However, the physiological function of this protein is still under investigation. NAMPT has been shown to have both intra- and extracellular enzyme activity, leading to the terms iNAMPT and eNAMPT respectively. It is suggested that eNAMPT acts as a cytokine, but whether this is discernible from its role in NAD biosynthesis is yet to be elucidated, and some conflicting data have been presented [22,26,171]. Currently, NAMPT research occurs in a

variety of fields, including NAD biology, metabolism and inflammation. Current knowledge of the functions of NAMPT in each of these areas is discussed in more detail in sections 1.6 - 1.8.

1.5.3 NAMPT gene and protein structure

The NAMPT gene is composed of 11 exons and 10 introns, and spans a length of 34.7 kb on Chromosome 7. Exon 1 encodes a short 5' untranslated region (UTR) and the signal peptide region, while exon 11 encodes a 3' UTR [176]. Samal *et al*. identified 3 NAMPT mRNA transcripts by northern blot analysis, of 2.0, 2.4 and 4.0 kb, the 2.4 kb species being the most abundant [167]. The NAMPT gene has two promoter regions, proximal and distal, in the 5' flanking region which contain multiple transcription initiation sites and multiple binding sites for transcription factors such as SP-1, NF-1, AP-1 and -2, STAT and NF-κB (Fig. 1.4). Some transcription factor binding sites are constrained to either the proximal or distal promoter; for example the SP-1 binding site is found solely in the proximal region and the NF-κB site solely in the distal region, whereas others, such as the AP-1 and STAT sites, are distributed throughout the promoter region. Both promoter regions contain putative regulatory elements responsive to hormonal and chemical signals, but the distribution has led to the suggestion that the proximal region is more susceptible to regulation by phosphorylation and hormones [23,176].

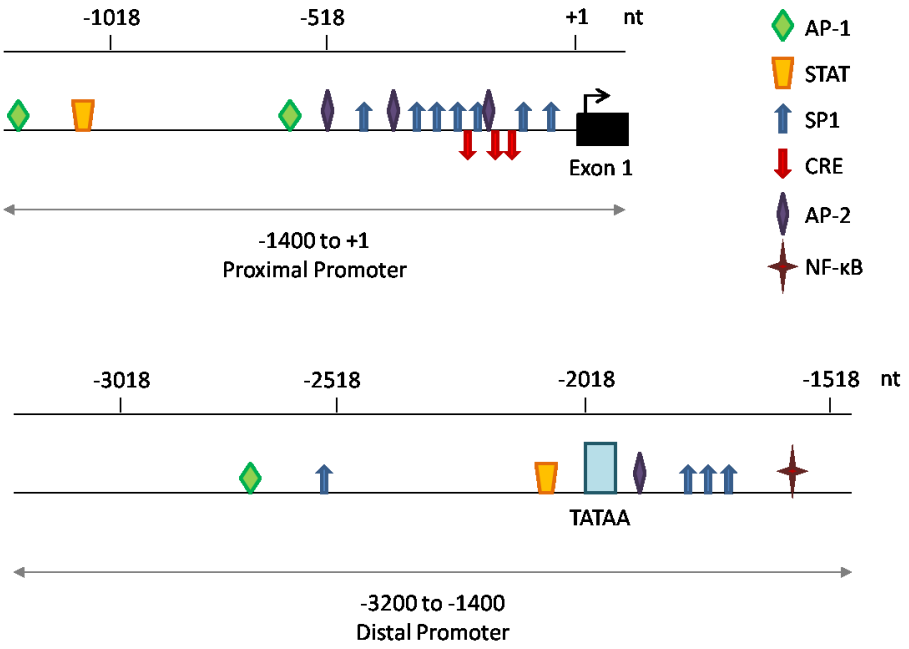

FIGURE 1.4: Schematic representation of the regulatory elements of the NAMPT promoter region. The 5' upstream region can be divided into the proximal (–1400 to +1) is more GC-rich, and the distal (–3200 to –1400) has more AT bases. The line marked nt (nucleotides) represents the distance of the regulatory element from the transcription initiation site, which is marked by an arrow in exon 1 (adapted from [23]).

A number of single nucleotide polymorphisms (SNPs) have been identified in the NAMPT gene, but the functional consequences of most of these SNPs are yet to be identified. However, a number of studies have linked NAMPT SNPs to serum levels of cholesterol, triglycerides, fasting insulin and glucose [177–181] and also susceptibility to acute respiratory distress syndrome (ARDS) [182,183]. NAMPT is expressed ubiquitously in nearly all tissues of the body [18,20,167,170,184], and is particularly highly expressed in the liver, peripheral blood leukocytes and adipose tissue [27,31,167,184]. Abnormal NAMPT expression has been associated with a number of disorders, especially those involving chronic inflammation [19,32,165,170,172,185–187].

The NAMPT 2.4 kb mRNA species encodes the 491 amino acid, 52 kDa NAMPT protein. The three-dimensional structure of mammalian NAMPT confirms it is a dimeric type II phosphoribosyltransferase (PRT), and despite low sequence identity with other type II PRTs, NAMPT shows significant tertiary structural similarity to PRT enzymes in other NAD biosynthesis pathways, such as nicotinic acid PRT and (NaPRTase) and quinolinic acid PRT (QaPRTase), although the active site topology differs between these enzymes [29,175]. NAMPT functions as a homodimer, with the active site formed at the dimeric interface and it binds nicotinamide (NAM), which with 5-phosphoribosyl-pyrophosphate (PRPP), is converted into nicotinamide mononucleotide (NMN) [29,175]. This is the rate-limiting step in the mammalian NAD salvage pathway [27,30], which is discussed in more detail in the following section. An inhibitor of NAMPT function, FK866 (also known as APO866 and WK175), has been developed which binds in a tunnel at the interface of the NAMPT dimer and competes directly with the substrate nicotinamide [28,29].

1.6 NAMPT in NAD Synthesis

NAMPT has been definitively characterised as a nicotinamide phosphoribosyltransferase that is crucial in the mammalian NAD salvage pathway [26,30]. NAD is a crucial metabolic coenzyme in redox reactions, and also serves as a substrate for NAD-consuming enzymes in a variety of biological processes. It remains unclear whether the proposed signalling

actions of NAMPT are due to a distinct cytokine-like function of this protein, or if these functions are attributed to the effects of modulating NAD production.

1.6.1 NAD

Nicotinamide adenine dinucleotide (NAD) is found in all living cells, as it has a central role in cellular metabolism and energy production. NAD and its phosphorylated (NADP) and reduced forms (NADH, NADPH) are crucial coenzymes in many redox reactions, readily accepting and donating electrons, as summarised in the equation below. This often leads to the production of ATP in dehydrogenase-catalysed reactions [188,189].

$$RH_2 + NAD^+ \rightarrow NADH + H^+ + R$$

More recently, however, NAD has been shown to be important as a substrate for NAD consuming enzymes that are involved in a range of biological processes [188]. They can be grouped into three general classes that utilise NAD as a substrate for 1) ADP-ribose transfer, 2) cADP-ribose synthesis or 3) protein deacetylation.

1) ADP-ribose transferases (ARTs) and poly-ADP-ribose polymerases (PARPs) cleave NAD-producing NAM and an ADP-ribosyl product. The latter is utilised by these enzymes for protein modification, which is important in processes including DNA

repair, transcriptional control, epigenetic modifications, G-protein coupled signalling and apoptosis [190–192].

2) cADP-ribose synthases catalyse production of cyclic ADP-ribose from NAD [193] and nicotinate adenine dinucleotide phosphate (NaADP) from NADP, both involved in calcium mobilisation [193,194].

3) Mammalian sirtuins are histone deacetylases (HDAC) that function in chromatin remodelling, which affects control of transcription. Sirtuins simultaneously bind acetylated lysine residues and NAD, and can deacetylate the residue by transferring the acetyl group onto the ADP-ribose moiety produced when NAD is cleaved [195,196].

In metabolic redox reactions, NAD is interconverted between its oxidised and reduced forms without net consumption. However, in these regulatory cellular processes where NAD functions a donor of ADP-ribose, it is cleaved. Because of this, NAD resynthesis is required to replenish intracellular stores. The ART, PARP, cADP ribose synthase and sirtuin enzymes that cleave NAD, can also be inhibited by nicotinamide that is released during cleavage [188,197–199]. Therefore, removal or salvage of nicotinamide is also a crucial stage in NAD resynthesis. NAD turnover within the cell is a dynamic process, as the half-life of the NAD molecule is estimated at 1-2 h [200]. NAD can be phosphorylated by the cytoplasmic NAD kinase (NADK), and NADP/H also functions as an electron

donor/acceptor in redox reactions, including the respiratory burst of neutrophils [201].

1.6.2 NAD Synthesis

Mammalian NAD is synthesised from a combination of *de novo* and salvage pathways (Fig. 1.5). *De novo* synthesis occurs in the liver where the essential amino acid tryptophan is converted into quinolinic acid via the so-called kynurenine pathway, which can then be converted into nicotinic acid mononucleotide (NaMN) [202]. At this point, the *de novo* pathway converges with the NAD salvage pathway(s). NAD can be recycled from nicotinic acid (Na) or nicotinamide (NAM). Lower eukaryotes predominately use Na as the NAD precursor, via the so-called Preiss-Handler pathway [203], and whilst this pathway exists in mammals they primarily synthesise NAD from nicotinamide. NAMPT catalyses the condensation of nicotinamide and 5-phosphoribosyl-pyrophosphate (PRPP) to form NMN (as summarised in the equation below), which is then converted into NAD by NMN adenyltransferase (NMNAT) [27]. There are three distinct isoforms of NMNAT (NMNAT1-3) which are localised to different subcellular compartments, namely the nucleus, cytoplasm and mitochondrion [204].

$$\text{NAM} + \text{PRPP} \xrightarrow{NAMPT} \text{NMN} + \text{PPi}$$

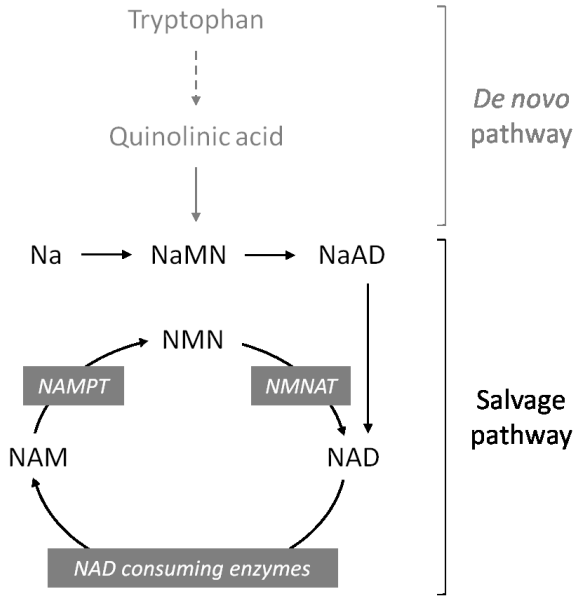

FIGURE 1.5: Mammalian NAD synthesis pathways. NAD can be synthesised *de novo* from tryptophan or recycled via the salvage pathways from either nicotinic acid (Na), via Na mononucleotide and Na adenine dinucleotide; or from nicotinamide (NAM) via nicotinamide mononucleotide (NMN). NAMPT catalyses the conversion of NAM to NMN, and the cycle is completed by the action of NMN adenyltransferase (NMNAT).

Nicotinamide represents the major source of NAD for most mammalian cell types, and the recycling of nicotinamide that is released during NAD cleavage by NAD-consuming enzymes, is an important homeostatic mechanism. NAMPT is the rate-limiting enzyme in the nicotinamide salvage pathway, and as such represents an important mediator of many cellular processes.

1.6.3 NAMPT inhibitor – FK866

The enzymic action of NAMPT can be blocked by the chemical inhibitor FK866 (also known as APO866 and WK175). FK866 was developed as an antitumor agent, as it was found to gradually deplete intracellular NAD,

which consequently led to apoptosis in the human monocytic cell line THP-1 [205]. Later, it was confirmed that FK866 depleted NAD and triggered apoptosis in HepG2 liver carcinoma cells, by inhibiting the NAMPT enzyme with an IC_{50} of approximately 1 nM. This study also calculated the inhibitor constants of 0.4 nM for the enzyme/substrate complex (K_i) and 0.3 nM for the free enzyme (K_i'), respectively [206]. This inhibition is specific to NAMPT and the nicotinamide salvage pathway, and FK866 does not affect the action of the closely-related enzymes nicotinic acid phosphoribosyltransferase (NaPRTase) and quinolinic acid phosphoribosyltransferase (QaPRTase) [28,206]. Kinetic studies led to the initial suggestion that FK866 inhibited NAMPT in a non-competitive manner [206], however more recently resolution of the crystal structures of NAMPT alone and in complex with the reaction product (NMN) or FK866 indicated that FK866 should compete directly with the nicotinamide substrate [28]. The authors suggest that the K_i of FK866 of 0.3 nM indicated that it may have a very slow dissociation rate from the enzyme, and that FK866 essentially functions as an irreversible inhibitor of NAMPT [28]. The resolution of the NAMPT crystal structure also confirmed that NAMPT functions as a dimer, as the active site is located in the dimer interface with residues from both monomers contributing to recognition of the substrate. The two monomers interact closely, with 4,000 $Å^2$ of the surface area of each in contact at the interface of the dimer. The dimer forms a tunnel at the monomer interface in which the active site is located. This tunnel is

about 15 Å and is also narrow, decreasing to 6 Å in places; this may account for the relatively slow dissociation of the inhibitor form the enzyme observed [28].

FK866 has recently completed phase II clinical trials for a number of solid and hematologic malignancies, and tumour cells are thought to be particularly sensitive to the effects of NAD depletion by FK866 due to their increased metabolic requirements. The use of this compound *in vitro* and in *in vivo* models has allowed manipulation of NAD turnover, and has increased understanding of the role of the NAD salvage pathway in many cellular processes. More recent work with FK866 has indicated that it also shows promise as an anti-inflammatory agent [19,33,34], suggesting that NAD is a key regulator of immune cell function.

1.6.4 Role of NAMPT as a NAD biosynthetic enzyme in human diseases

Dysregulation of NAD homeostasis can be causative or indicative of numerous disorders, as NAD(P) participates in a wide array of metabolic and other fundamental cellular pathways. As NAMPT is the major enzyme that controls the rate of NAD synthesis, it is also implicated in many key cellular processes. In fact dysregulation of NAMPT function has been implicated in a number of human diseases and conditions, such as acute respiratory distress syndrome [182,183], aging [204,207], atherosclerosis [185], inflammatory bowel disease [20], cancer [206,208,209], diabetes [172,210], sepsis [19] and rheumatoid arthritis [18,32,33,165]. Although NAMPT acting as a cytokine

has been implicated in many of these disorders, its effect on many, if not all, of these conditions can also be attributed to availability of NAD either for specific NAD-consuming enzymes, such as the sirtuins (in aging and PARPs in DNA repair and cancer), or for metabolic processes such as the respiratory burst of immune cells. The role of NAMPT as an enzyme in inflammatory disease, specifically rheumatoid arthritis, will be covered in section 1.8.

1.7 NAMPT as a Cytokine

NAMPT is a ubiquitously-expressed protein, and has been reported to exert a variety of effects on a number of cell types. It is purported to have a cytokine-like function, and was initially identified by the sequence similarity in its coding region to that of other cytokines [167]. Exogenous NAMPT can stimulate expression of cytokines, chemokines and matrix-degrading enzymes from synovial fibroblasts and monocytes [18], and it delays the apoptosis of neutrophils and macrophages [19,22]. However, the mechanism by which NAMPT is released from cells is still a matter of debate [211,212], and no cell surface receptor for NAMPT has been identified. It was suggested that NAMPT can bind to the insulin receptor at a site distinct from that of insulin [31], although this has not been reconfirmed to date [26]. The proposed role of NAMPT as a cytokine in inflammatory and metabolic diseases is discussed in this section.

1.7.1 NAMPT expression and release

NAMPT is essential for development, as it has been shown that NAMPT gene knockouts are embryonic lethal in mice [26,31,174]. NAMPT is ubiquitously expressed in the body, although it is particularly highly-expressed in the liver, adipose tissue and immune cells. It is well-validated that NAMPT expression can be upregulated in a number of immune cells in response to endotoxin and inflammatory cytokines [20]. The expression of NAMPT in response to these stimuli, in a variety of cell types, is summarised in Table 1.3.

NAMPT is detected at relatively high levels in the circulation (~90 ng/mL), at a concentration around 9 times that of other common adipokines such as leptin, adiponectin and resistin [165]; cytokines such as TNFα and IL-6 are generally observed to be around 10-20 pg/mL in the serum [214]. NAMPT is reported to be significantly elevated during inflammation [18,32,165], obesity and diabetes [31,172], amongst other conditions. However, it is still not well understood how NAMPT is released from cells, and some argue that it is released solely through cell death and lysis [171,212]. NAMPT does not contain a putative N-terminal signal sequence, commonly-seen in other secreted cytokines [167,176], but it has been suggested that NAMPT is actively-secreted by the 3T3-L1 adipocyte cell line during culture, in similar ways as other adipokines are secreted. [211]. This secretion was independent of the endoplasmic reticulum (ER)-Golgi system, as it was not

STIMULUS	CELL TYPE	REFERENCE
TNFα	Monocytes	Dahl *et al*., 2007 [185]
	Macrophages	Iqbal & Zaidi, 2006 [213]
	Neutrophils	Jia *et al*., 2004 [19]
	Amniotic epithelial cells	Ognjanovic *et al*., 2001 [176]
	Lung microvascular endothelial cells	Ye *et al*., 2005 [182]
IL-1β	Neutrophils	Jia *et al*., 2004 [19]
	Amniotic epithelial cells	Ognjanovic *et al*., 2001 [176]
	Lung microvascular endothelial cells	Ye *et al*., 2005 [182]
IL-6	Amniotic epithelial cells	Ognjanovic *et al*., 2001 [176]
	Synovial fibroblasts	Nowell *et al*., 2006 [32]
IL-8	Neutrophils	Jia *et al*., 2004 [19]
GM-CSF	Neutrophils	Jia *et al*., 2004 [19]
Oncostatin-M	Synovial Fibroblasts	Nowell *et al*., 2006 [32]
Endotoxin/LPS	Neutrophils	Jia *et al*., 2004 [19]
	Amniotic epithelial cells	Ognjanovic *et al*., 2001 [176]
	Lung microvascular endothelial cells	Ye *et al*., 2005 [182]

TABLE 1.3: Expression of NAMPT induced by inflammatory mediators in a number of cell types. Adapted from [23].

blocked by addition of inhibitors of this pathway, and microvesicles secreted by these cells contained very little NAMPT. Thus, it was suggested that secretion occurs via a non-classical pathway, consisting of several discrete secretory machineries [211]. One study has addressed the expression and release of NAMPT from neutrophils; it was shown that NAMPT mRNA transcripts were upregulated by exposure to LPS, TNFα and IL-1β, and conversely decreased by addition of pro-apoptotic stimuli (anti-CD95 antibody and heat-killed *Candida albicans*). NAMPT protein was also detected in the culture supernatant of LPS stimulated neutrophils [19].

1.7.2 NAMPT signalling

It has been shown that exogenous NAMPT can activate expression of IL-1β, IL-6 and TNFα in monocytes in a p38- and MEK-1-dependent manner; inhibition of p38 MAPK abrogates this NAMPT-induced cytokine expression [20]. It is also reported that exogenous NAMPT can activate NF-κB signalling in endothelial cells; NAMPT-mediated expression of matrix metalloproteinase-2/9 (MMP-2/9) from these cells was abrogated by addition of an NF-κB inhibitor [215]. In many of these studies of NAMPT cytokine-like activity, the role of NAMPT as an enzyme is not specifically addressed. Others, however, have attempted to reconcile these two supposed functions. For example, mutant recombinant NAMPT protein (with no enzyme function) was shown to stimulate expression of IL-8, IL-

16 and CCR3 in human pulmonary epithelial cells but did not trigger NAD biosynthesis. It was identified that the transcription factor, AP-1, was required to mediate these effects, and MAPK and JNK inhibitors attenuated the activity [21]. In other studies, the enzyme function of NAMPT did appear to be crucial for signalling. For example, in human vascular smooth muscle cells, NAMPT was shown to induce sustained activation of NF-κB and biphasic activation of ERK1/2, leading to enhanced iNOS production. The activation of these signalling pathways by NAMPT was abrogated by addition of the NAMPT enzyme inhibitor FK866, and was mimicked by addition of the NAMPT enzyme product nicotinamide mononucleotide (NMN) [216].

Exogenous NAMPT is also reported to have an anti-apoptotic effect on neutrophils and macrophages [19,22]. This anti-apoptotic effect resulted in decreased activation of caspases-3 and -8, but not caspase-9, suggesting that NAMPT targets the extrinsic apoptotic pathway. It was shown that endogenous intracellular NAMPT was required for this process, but the role of NAMPT as an enzyme in this process was not discussed [19]. In macrophages, NAMPT was shown to block ER-stress induced apoptosis via a two-step process that involved initial rapid induction of IL-6 secretion, followed by IL-6 mediated activation of STAT3. This effect was not altered by addition of the NAMPT enzyme inhibitor FK866, or by use of site-directed mutant NAMPT proteins [22].

Current opinion still favours the dual-role hypothesis of NAMPT, activating cells like a cytokine in some circumstances and affecting cellular function via mediation of NAD biosynthesis and metabolism in others. It is clear that exogenous NAMPT can induce stimulatory effects on various cell types, but whether this is due to NAMPT induced expression of other cytokines and subsequent autocrine/paracrine activation of signalling pathways, is yet to be determined.

1.8 NAMPT in Inflammatory Disease

NAMPT expression is increased in a number of acute and chronic inflammatory diseases, including sepsis [19], inflammatory bowel disease [20,217], acute lung injury [182], atherosclerosis [185] and rheumatoid arthritis (RA) [18,32,165,218]. A number of studies have recently described the potential of NAMPT inhibition (with FK866) to modulate the immune response, and it has shown promise in alleviating the symptoms in mouse models of inflammatory arthritis [33,34].

1.8.1 NAMPT in rheumatoid arthritis

It is well established that NAMPT expression is elevated in the serum and synovial fluid of RA patients [18,165,218], and levels are significantly higher than those seen in osteoarthritis (OA) patients [32]. It is also elevated in other inflammatory conditions, such as Beçhet's disease, and juvenile idiopathic arthritis (JIA) [219]. Serum NAMPT is associated with radiological joint

damage in RA, even when other inflammatory mediators such as TNFα, IL-6 and CRP are considered [18,165,220,221], suggesting that NAMPT is independently associated with RA. Some studies suggest that NAMPT correlates with clinical measurements of disease in RA, such as serum CRP and DAS [18], but others did not find a correlation with DAS [220].

NAMPT is elevated in synovial tissue during inflammation in RA [218], specifically in a number of structural cells of the synovial joint, including chondrocytes, synovial fibroblasts, endothelial cells and fibroblast- and macrophage-like synoviocytes [18,32,34]. It has been demonstrated that NAMPT is highly expressed in fibroblast-like synoviocytes, specifically localised at points of invasion into the synovial lining and cartilage [18]. Some studies suggest that synovial lining fibroblasts and synovial lymphoid aggregates may provide the major source of NAMPT expression in the joint [18,32]. Expression of NAMPT in cells of the rheumatoid joint can be regulated by exposure to cytokines, for example IL-1β stimulates NAMPT expression in chondrocytes [222], and it is stimulated in synovial fibroblast-like cells by addition of IL-6 and its soluble receptor sIL-6R, in a STAT3-dependent manner [32,34]. Addition of exogenous NAMPT has also been shown to activate NF-κB and AP-1 in synovial fibroblasts, subsequently promoting expression of IL-6, IL-8, CCL2 and MMP-1 and -3 [34].

NAMPT is also expressed by a number of the immune cell types involved in inflammatory arthritis, such as neutrophils, monocytes [19], macrophages, dendritic cells [33], and T and B lymphocytes [167,223] (Table 1.3). During inflammation, it is suggested that peripheral blood leukocytes are a major source of circulatory NAMPT, as serum NAMPT correlated with white blood cell count in one study of children [184]. Another study (in RA adults) confirmed that serum NAMPT correlated with mRNA expression from PBMCs [218], and it is considered one of the only universal markers of inflammation in these cells [217]. Lymphocytes and macrophages are also major sources of NAMPT during chronic inflammation, and its expression is upregulated in response to inflammatory mediators. Continuous activation of these cells during chronic inflammation leads to a high level of NAMPT in the circulation and in the joints infiltrated by these immune cells during RA.

Exogenous NAMPT activates a number of immune cell types and stimulates expression other cytokines and chemokines, perpetuating the immune response. For example, monocytes stimulated with NAMPT are activated to secrete a variety of inflammatory mediators such as TNFα, IL-6 [18], IL-1β, IL-1Ra and IL-10, alongside an increase in the surface expression of CD40, CD54 and CD80 [20]. Neutrophils are the most abundant cell type found in the rheumatoid joint, and have the greatest capacity for tissue damage [11]. Transcription of NAMPT is induced in

neutrophils by IL-1β [19] and NAMPT has been described as exacerbating the pro-inflammatory action of these cells in a number of ways. One study suggests that NAMPT primes neutrophils for an augmented respiratory burst via partial assembly of the NADPH oxidase complex, leading to production of ROS upon further stimulation [224]. NAMPT has also been shown to delay neutrophil apoptosis, exacerbating the inflammatory state further. In fact, antisense oligonucleotide knock-down of NAMPT expression abolishes the anti-apoptotic action of TNFα, GM-CSF, IL-1β and IL-8 suggesting that NAMPT is crucial to the regulation of neutrophil apoptotic mechanisms [19].

Considering the evidence, it appears that NAMPT is involved in the dysregulated immune response observed during chronic inflammation and joint destruction in RA. As the concentration of NAMPT in the inflamed synovial joint correlates with rheumatoid joint damage, it likely contributes to the destructive inflammatory process, either directly through activation of immune cells or by stimulating expression of other pro-inflammatory cytokines. Analysis of RA tissues lends credence to this hypothesis and together this evidence has led to the proposal that NAMPT may be an attractive therapeutic target in inflammatory disease.

1.8.2 Effects of NAMPT inhibition on the inflammatory process

A number of studies have expanded upon the potential of NAMPT as a therapeutic target in inflammatory disease, by investigating the effects of

NAMPT inhibition on these processes. Existing therapies for rheumatoid arthritis have been shown to impact NAMPT concentration in the circulation; RA and JIA patients showed decreased circulatory NAMPT following both anti-TNF (infliximab) and methotrexate treatment [225,226], and B-lymphocyte depletion therapy for RA causes a similar decrease in serum NAMPT [227]. New ideas for therapies are emerging that target the actions of this molecule directly, by inhibiting its function as an enzyme of the NAD biosynthetic pathway. The role of NAMPT in NAD biosynthesis may also be important in inflammation, as NAMPT regulates the function of the sirtuin proteins which are involved in the transcriptional control of many cellular processes including the cellular response to cytokines [169]. Van Gool *et al*. [35] identified that NAMPT is responsible for the upregulation of intracellular TNFα via the NAD-dependent enzyme Sirt6. They, amongst others, also confirmed that an accumulation of nicotinamide (the substrate of NAMPT) efficiently down-regulated cytokine production when not converted to NMN by NAMPT [228]. FK866 also inhibited the NAMPT-induced increase in expression of MMP3, CCL2 and IL-8, and decreased the activity of MMP3, CCL2 and RANKL [34]. Activated T-lymphocytes are also acutely affected by NAMPT inhibition, undergoing significant NAD depletion and decreased expression of IFNγ and TNFα, before undergoing autophagic cell death [223]. *In vitro*, FK866 significantly decreased the secretion of TNFα, IL-1β and IL-6 from monocytes upon stimulation with bacterial agents, which was reversed by addition of the

NAMPT enzyme product NMN [33]. FK866 has also been reported to decrease production of ROS by monocytes in response to stimuli [229], and this may be attributed to the effects of NAD depletion on the action of the NADPH oxidase. However, it has also been shown that accumulation of nicotinamide, the substrate of NAMPT, has the potential to suppress production of reactive oxidants [230].

Due to the proposed down-regulation of immune cell activation, production of inflammatory mediators and release of tissue-damaging products, NAMPT inhibition is an attractive option for treatment of chronic inflammatory disease. Two studies have assessed the potential of FK866 to modulate disease in the collagen-induced arthritis (CIA) mouse model. Busso *et al*. [33] reported that injection of FK866 into CIA mice decreased serum TNFα levels and the severity of the arthritis, to an extent comparable to the anti-TNF drug etanercept [33]. In a similar study by Evans *et al.* [34], early intervention with FK866 in CIA mice inhibited synovial inflammation and leukocyte infiltration, and protected against bone erosion. These mice also showed decreased serum hyaluronic acid, a marker of systemic inflammation. Joint extracts showed that FK866 treatment decreased expression of MMP-3 and -13 and markers of cartilage erosion. In mice with established disease FK866 treatment led to decreased synovial inflammation and cartilage destruction and also prevented bone erosion [34]. Both of these studies in mice reported no adverse toxicity of

FK866. Interestingly they both reported that FK866 treatment had no effect on the levels of autoantibodies against type II collagen, suggesting that the impact of FK866 is mainly mediated through the local disruption of the destructive processes in the joint [33,34]. These beneficial effects may be due to the initial down-regulation of cytokines and destructive enzymes in the joint. Studies into the feasibility of NAMPT inhibition as a viable therapy for RA are ongoing, but these early results showing a concurrent decrease in pro-inflammatory molecules and in the destructive processes within the joint, appear promising. FK866 has recently completed phase II clinical trials for the treatment of solid and haematological malignancies, and has demonstrated a good safety profile, suggesting that it may be a viable future treatment option in RA, either alone or alongside existing therapies [231,232].

1.9 Summary

Neutrophils have a pathologically important role in the progression of RA. Inappropriately primed neutrophils infiltrate rheumatoid joints in vast numbers and become activated, resulting in delayed apoptosis and release of tissue damaging products [2,6,11]. RA is a progressively degenerative inflammatory joint disease, so targeting the cells that mediate the damage would be expected to be beneficial.

During a transcriptomics screen for potential inflammatory biomarkers in neutrophils, high levels of NAMPT mRNA expression were identified in

neutrophils from patients with inflammatory disease. NAMPT has been previously implicated in inflammatory disease, and correlates with clinical measurements of disease in RA [18]. It is unclear if one particular function of NAMPT, which is proposed to act both as a cytokine and as an enzyme in NAD biosynthesis, is important in inflammation. However, the ability of NAMPT to activate immune cells, stimulating expression of cytokines and destructive enzymes, coupled with a role in mediating neutrophil apoptosis, place NAMPT as an important mediator of inflammation [19–24]. This, alongside the down-regulation of key inflammatory mediators, and alleviation of disease severity observed in mouse models of inflammatory arthritis reported with NAMPT inhibition (with FK866) [33–35], suggest that NAMPT is an attractive therapeutic target in RA.

1.10 Research Aims

The overarching aim of this research was to better understand the role of neutrophils in inflammation, using RA as a model disease. Previous work has identified NAMPT as a potential biomarker of inflammation in neutrophils, so more specifically the aim of this thesis was to understand the role of NAMPT in neutrophil functions and gene expression. One particular aim was to evaluate whether this molecule could be a viable therapeutic target in inflammatory disease.

The specific aims of the research were to:

1. Evaluate whether the role of NAMPT in NAD production is of importance to neutrophil function; to determine the level of NAD, and its phosphorylated and reduced forms in the cell, and observe the effects of NAMPT enzyme inhibition and NAD depletion on neutrophil function and apoptosis.

2. Determine whether exogenous NAMPT is capable of priming neutrophils and augmenting their function in a manner similar to that of other cytokines.

3. Investigate the expression and release of NAMPT in both resting neutrophils and in response to key inflammatory cytokines, and comparing this to NAMPT expression from the neutrophils of RA patients.

4. Determine the effects of both NAMPT stimulation and NAMPT inhibition on neutrophil intracellular signalling pathways and mRNA expression profiles.

CHAPTER 2: Materials and Methods

2.1 Materials

Cytokines, Stimulants and Inhibitors	Supplier
Recombinant human TNFα	Calbiochem (Nottingham, UK)
Recombinant Human GM-CSF (50,000 U)	Roche Diagnostics Ltd. (East Sussex, UK)
Recombinant human IL-8 Recombinant human IL-1β	Invitrogen (Paisley, UK)
Recombinant human NAMPT* Cytochalasin B (CB) N-formylmethionyl-leucyl-phenylalanine (fMLP) Phorbol 12-myristate 13-acetate (PMA) Recombinant human IL-6 Bacterial lipopolysaccharide (LPS)	Sigma (Poole, UK)
FK866 BAY-11-7082	Cambridge Bioscience (Cambridge, UK)

TABLE 2.1

*Full length recombinant NAMPT was expressed in *E. Coli* and following purification was determined by Sigma to be of ≥95% purity by HPLC and SDS-PAGE analysis. The endotoxin level was also determined to be <0.1 EU per μg of NAMPT (LAL test).

Media and Chemicals	Supplier
Glycerol Sodium dodecyl sulphate Tris	Fisher Scientific (Loughborough, UK)

Hanks balanced salt solution (HBSS) RPMI 1640 (+ 25 mM HEPES, 2 mM L-glutamine) Trizol® reagent	Gibco (Paisley, UK)
MTS reagent (Cell titer non-radioactive-proliferation assay kit)	Promega (Southampton, UK)
Phosphate buffered saline (PBS) tablets	Oxoid Ltd (Basingstoke, UK)
30% Hydrogen peroxide (H_2O_2) Ammonium chloride (NH_4Cl) Ammonium persulphate (APS) Bovine serum albumin (BSA) Dethylpyrocarbonate (DEPC) Dithiothreitol (DTT) Dimethyl sulfoxide (DMSO) Ethanol (general laboratory grade) Ethylenediaminetetracetic acid (EDTA) Ferri-cytochrome c Glycine Horseradish peroxidise (HRP) Human pooled AB serum Isoluminol Luminol (disodium salt) Paraformaldehyde Sodium azide Tetramethylethylenediamine (TEMED) Tween-20	Sigma (Poole, UK)
Hydrochloric acid (HCl)	VWR International (Leicestershire, UK)
Polyacrylamide	Severn Biotech (Kidderminster, UK)

TABLE 2.2

Antibodies, dyes and probes	Supplier
HRP-conjugated donkey anti-rabbit IgG	Amersham Life Sciences (Bucks, UK)
Fluorescein isothiocynate (FITC)-conjugated mouse anti-human CD16 FITC-conjugated anti-human CD32	BD Biosciences (Cowley, UK)
Rabbit anti-human lactoferrin Sheep anti-human myeloperoxidase	Biodesign International (Kennebunk, USA)
HRP-conjugated rabbit anti-sheep IgG	Dako (Glostrup, Denmark)
FITC-conjugated Annexin V	Invitrogen (Paisley, UK)
Allophycocyanin (APC)-conjugated rat anti-human CD11b	Miltenyi Biotec (Surrey, UK)
Bromophenol blue HRP-conjugated sheep anti-mouse IgG Ponceau S Propidium iodide (PI)	Sigma (Poole, UK)
Mouse anti-human Actin	Abcam (Cambridge, UK)
Mouse anti-human extracellular TNFα-FITC FITC-conjugated anti-human L-selectin	R&D Systems (Abingdon, UK)

TABLE 2.3

Other Materials	Supplier
Enhanced chemiluminescence hyperfilm	Amersham Life Sciences
Polymorphprep™	AxisSheild (Cambs, UK)
dNTPs	Bioline (London, UK)
Biotinylated protein ladder detection pack	Cell-Signalling Technology (Massachusetts, USA)

Primers	Eurofins MWG Operon (Ebersberg, Germany)
Lithium heparin vacutainers	Grenier Bio-one (Gloucestershire, UK)
Rapid Romanowsky stain	HD Supplies (Aylesbury, UK)
Superscript III first strand cDNA synthesis kit RNase OUT	Invitrogen (Paisley, UK)
Immobilon western chemiluminescence HRP-substrate Polyethylene Terephthalate (PET) millicell 3 μm hanging inserts Polyvinylidene fluoride (PVDF) membrane	Millipore (Hertfordshire, UK)
Random primers	Promega (Southampton, UK)
RNeasy mini kit Quantitect SYBR green PCR kit	Qiagen (Crawley, UK)
Staphylococcus aureus	Donated by department of Medical Microbiology, University of Liverpool, UK
Marvel non-fat powered milk	Tesco
Slide-A-lyzer 10 k dialysis cassettes (3-5 mL) BCA protein assay	Pierce Technology (Northumberland, UK)

TABLE 2.4

2.2 Methods

2.2.1 Neutrophil isolation from whole blood

Ethical approval for the study of neutrophils from healthy control individuals was obtained from the University of Liverpool Committee for Research Ethics (CORE), reference RETH000398. Ethical approval for the study of neutrophils from patients was obtained from the North West 3 (Liverpool East) Research Ethics Committee, study number 07/Q1502/4. All blood donors gave informed consent. Blood was collected into heparinised vacuette tubes, by venupuncture of healthy volunteers and RA patients. Heparin was included to prevent red blood cell coagulation, as red blood cells are crucial to the generation of a density gradient during neutrophil isolation with Polymorphprep. Since neutrophils are short-lived and should be used within a few hours of collection, a fast and reliable method of separating them from other blood cells is desirable, to enable robust assay of neutrophil function *in vitro.* Polymorphprep is currently the only commercially available medium capable of separating PBMC and neutrophils from whole blood in one step. Other methods such as dextran-sedimentation followed by Ficoll-Paque isolation are much more time-consuming. However, the efficacy of the Polymorphprep method requires fresh blood, <2 h from collection [233]. Polymorphprep relies on an isoosmotic density barrier that separates the mononuclear cells and neutrophils into two distinct bands during centrifugation, whilst the red

blood cells settle to the bottom of the solution[233]. This allows immediate aspiration of the neutrophil layer. Here, Neutrophils were isolated from whole blood by one-step centrifugation with Polymorphprep (as described in the manufacturer's instructions). Briefly, blood was layered onto Polymorphprep at a 1:1 to 1.5:1 ratio, and centrifuged at 500 g for 35 min. Granulocytes were removed and resuspended in RPMI 1640 media then centrifuged at 500 g for 5 min. Cells were resuspended in media and contaminating erythrocytes were removed by ammonium chloride lysis (155 mM NH_4Cl_4, 13.4 mM $KHCO_3$, 97 μM EDTA, pH 7.6); red blood cell membranes are permeable to ammonium chloride, and it causes cell lysis due to an imbalance in the osmotic pressure. Neutrophils are widely reported to be unaffected by ammonium chloride treatment, but some affects on iodination have been reported over time, so here lysis was performed for 3 min [234]. Any red blood cells remaining following centrifugation were at the top surface of the neutrophil pellet and so were washed away with a rinse of medium. Neutrophils were resuspended in RPMI 1640 (with 25 mM HEPES, 2 mM L-glutamine), and adjusted to a final concentration of 5 x 10^6 cells/mL (unless otherwise stated) using a Multisizer 3 cell counter (Beckman Coulter). Final neutrophil purity was routinely greater than 97% by cytospin. Cells were incubated with gentle agitation at 37°C, and where indicated 10% AB serum was added for incubations >4 h. Cytokines and inhibitors were added as indicated.

2.2.2 Assay of neutrophil NAD content

Neutrophils in culture at 5 x 10^6 cells/mL were centrifuged at 1000 g for 3 min then lysed with 250 µL of 1% Triton/PBS (v/v) per 5 x 10^6 cells, for 15 min on ice. Samples were then centrifuged at 5000 g for 3 min to remove cell debris, and supernatant was aspirated for use in the NAD(P)H assay. Cellular NAD(P)H content was assayed using the Amplite™ Colormetric NAD/H or NADP/H assay kit from AAT Bioquest as per manufacturer's instructions. Briefly, 50 µL of sample or NAD(P)H standard was mixed with the corresponding 50 µL NAD(P)H reaction mixture in a 96-well white (clear bottom) microtitre plate, and incubated for 1 h protected from light. The absorbance was assayed on a Bio-Rad 3550 microplate reader at 570 nm with the reference wavelength set at 620 nm. The cellular NAD(P)H concentration was determined by comparison to NAD(P)H standards of known concentration and adjusted for the number of cells.

The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega) was also used as an indirect measure of total cellular NAD(P)H. Briefly, 100 µL neutrophils (5x10^6 cells/mL) in culture medium were added to 20 µL of CellTiter 96® AQueous One Solution Reagent in a 96-well microtitre plate, and incubated for 4 h in a humidified 5% CO_2 atmosphere protected from light. Relative NAD(P)H content was assayed by absorbance at 490 nm.

2.2.3 Flow cytometric analysis of neutrophil surface marker expression

For measurement of neutrophil surface marker expression, 1 x 10^6 cells were fixed in 2% (w/v) paraformaldehyde (PFA) for 10 min at room temperature. Where indicated, cells were then permeabilized with 0.04% (w/v) saponin with PBS (0.1% (w/v) BSA), and 0.1 M glycine. Following washing with PBS/BSA, the cells were incubated for 30 min at 4°C with 5 µL of the indicated fluorescently-conjugated antibody. 150 µL of PBS/BSA was then added to the cells, and the total volume was added to a 96-well microtitre plate for flow cytometry. All samples were analysed using the Guava Easycyte flow cytometer (Miltenyi Biotech), and a minimum of 5000 gated events were collected.

2.2.4 Flow cytometric analysis of neutrophil apoptosis

Neutrophil apoptosis was quantified by flow cytometry as the percentage of cells exhibiting external expression of membrane phosphatidylserine, detected by annexin V binding, and necrosis was quantified by propidium-iodide (PI) uptake due to membrane permeabilisation. 1 x 10^5 cells in 100 µL HBSS were incubated with 1 µL annexin V for 30 min at room temperature, protected from light in a 96-well microtitre plate. Following this, 1 µL PI (1 µg/mL) was added just prior to analysis by flow cytometry. Neutrophil viability was also quantified using the Guava Viacount Reagent (Millipore), according to the manufacturer's instructions. The Viacount

reagent contains two DNA binding dyes of differing cell membrane-permeability. All nucleated cells are stained by the membrane-permeable dye, but the membrane-impermeable dye stains only cells with increased membrane permeability, indicating apoptotic or dying cells [235]. Briefly, 200 μL of the assay reagent was added to 10^6 cells in a 96-well microtitre plate, and immediately subjected to flow cytometry.

2.2.5 Neutrophil transmigration assay

A model of neutrophil transmigration was set up using Millipore Hanging Cell Culture plate inserts with a 3 μm pore-size filter at their base. To prevent neutrophil adherence following transmigration, 24-well tissue culture plates were coated with 400 μL poly-hema (12 mg/mL) in ethanol, which was left to evaporate at 37°C for at least 24 h. For the assay, 800 μL of culture medium plus the indicated chemoattractant was added into each well. The hanging cell inserts were then suspended in the media and left to equilibrate for 10 min at 37°C. Following this, 1 x 10^6 freshly isolated neutrophils in 200 μL culture media were added into the hanging insert, and incubated for 1 h at 37°C (Figure 2.1). The inserts were then removed, and the cells that had migrated through the membrane into the well beneath were counted using the Multisizer 3 cell counter (Beckman Coulter) following a 1:1000 dilution with Isoton II. Chemotaxis was determined as the number of migrated cells calculated as a percentage of the total number of cells added.

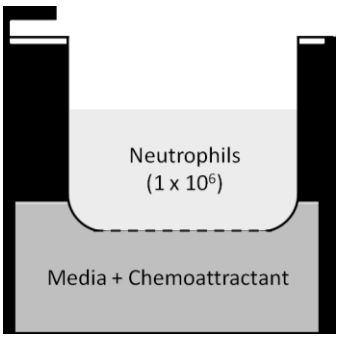

FIGURE 2.1: Hanging insert assay for neutrophil chemotaxis.

2.2.6 Preparation of neutrophil lysates for analysis of protein

Following culture, cells were sedimented at 1000 g for 3 min, and the cell pellet was rapidly lysed in reducing 1x Laemmli buffer, containing 10% (v/v) glycerol, 100 mM DTT, 3% (w/v) SDS, 0.001% (w/v) bromophenol blue and 1 M Tris-HCl (pH 6.8), to a final concentration of 5 x 10^4 cells/µL. For assessment of proteins released during culture, boiling 5x Laemmli buffer was added to the aspirated culture medium at a ratio of 5:1 supernatant to buffer. All samples were boiled for 3 min with occasional vortexing, and stored at -20°C until use. For concentration of low abundance proteins from the culture supernatant, 4 volumes of cold (-20°C) acetone were added to the supernatant and proteins were precipitated for at least 1 h at -20°C. The protein was sedimented by centrifugation at 13,000g for 10 min, and resuspended in 25 µL Laemmli buffer.

2.2.7 Western blotting

Whole cell extracts were loaded to into 8-12% polyacrylamide gels (dependent on molecular weight of target protein) to an equivalent of 0.5-2 x 10^6 cells per well, along with a biotinylated protein molecular weight marker and subjected to SDS-PAGE. Electrophoresis was performed for 1 hour at a constant voltage of 200V using BioRad Mini Protean II Electrophoresis Apparatus. Following this, proteins were electrotransferred to a PVDF membrane at a constant voltage of 100V for 90 min using the BioRad Mini Protean II Transfer Apparatus. Successful transfer of proteins was confirmed using Ponceau S staining (0.01% (w/v) in 5% (v/v) acetic acid). To prevent non-specific binding of the antibody, PVDF membranes were incubated with blocking buffer containing 5% (w/v) non-fat dried milk in wash buffer (Tris buffered saline (TBS) 1 mM Tris, 15 mM NaCl, pH 8.0, 0.075% (v/v) Tween-20), for at least 1 h at room temperature. Membranes were then incubated with the required primary antibody in blocking buffer at the indicated concentration (Table 2.5) overnight at 4°C. Following washing, the membranes were incubated with the appropriate HRP–conjugated secondary antibody in blocking buffer, for at least 1 h at room temperature. The membranes were washed again then bound antibodies were detected using enhanced chemiluminescence (ECL) reagents (Millipore) and exposure to Hyperfilm in the dark. Densitometry of blots was performed with AQM Advanced 6 Kinetic Imaging System.

Levels of β-actin were measured to confirm equivalent loading of protein in neutrophil samples.

Target protein	Primary antibody	Secondary antibody
Nampt	Rabbit anti-human Nampt (1:2500)	HRP-conjugated donkey anti-rabbit IgG (1:10000)
Myeloperoxidase	Sheep anti-human MPO (1:10000)	HRP-conjugated rabbit anti-sheep IgG (1:10000)
Lactoferrin	Rabbit anti-human lactoferrin (1:10000)	HRP-conjugated donkey anti-rabbit IgG (1:10000)
Mcl-1	Rabbit anti-human Mcl-1 (1:1000)	HRP-conjugated donkey anti-rabbit IgG (1:10000)
CD16b	Mouse anti- human FcγIIIb (3G8 cell line) (1:100)	HRP-conjugated sheep anti-mouse IgG (1:10000)
ph-NF-κB	Rabbit anti-human phospho-p65 (1:1000)	HRP-conjugated donkey anti-rabbit IgG (1:10000)
ph-ERK1/2	Mouse anti-human phospho- p44/p42 (1:1000)	HRP-conjugated sheep anti-mouse IgG (1:10000)
ph-STAT3	Mouse anti-human phospho- STAT3 (1:1000)	HRP-conjugated sheep anti-mouse IgG (1:10000)
IκBα	Rabbit anti-human IκBα (1:1000)	HRP-conjugated donkey anti-rabbit IgG (1:10000)
β-Actin	Mouse anti-human actin (1:10000)	HRP-conjugated sheep anti-mouse IgG (1:10000)
Biotin	n/a	HRP-linked anti-biotin

TABLE 2.5 Western Blot Antibodies

2.2.8 Isolation of neutrophil mRNA and cDNA synthesis

1 x 10^7 (3 x 10^7 for transcriptome sequencing) cells were sedimented at 1000 g for 3 min and lysed with 1 mL Trizol reagent. The pellet was disrupted by pipetting until a homogenous solution was obtained, which was then incubated for at least 5 min at room temperature. Following this, 200 µL of chloroform was added to the samples and mixed by inversion for 15 s, before incubating for 2-3 min at room temperature. The samples were then centrifuged at 10,000 g for 15 min at 4°C, and the resulting aqueous phase containing the RNA was removed, and mixed with an equal volume of isopropanol to precipitate the RNA, either for 20 min at room temperature or at -20°C overnight. The samples were then centrifuged at 10,000 g for 30 min at 4°C, to pellet the precipitated RNA, which was then washed in 70% ethanol, and resuspended in 100 µL RNase-free water. The RNA samples were cleaned to remove remaining contaminants using the Qiagen RNeasy kit according to the manufacturer's instructions, and genomic DNA was removed by a 15 min on-column digestion with Qiagen DNase. RNA was then eluted in 30 µL RNase-free water. The RNA content was quantified using the Nanodrop ND-1000 spectrophotometer. RNA samples were stored at -80°C until use.

cDNA synthesis was performed using the Superscript III First Strand cDNA Synthesis kit (Qiagen) and RNase OUT (Qiagen), according to the manufacturer's instructions, as per Table 2.6. RNA samples were adjusted

to the same concentration (<10 ng/μL) within each experiment prior to cDNA synthesis. cDNA samples were stored at -20°C until use.

Reagents Added	Temperature (°C)	Time (min)
RNA (<10 ng/μL) 11 μL Random Primers (20 μM) 1 μL dNTPs (10 mM) 1 μL	60	5
	0 (Ice)	>1
First-Strand buffer 5 μL RNase OUT 1 μL DTT (0.1 M) 1 μL Superscript III reverse transcriptase 1 μL	25	5
	50	60
	70	15

TABLE 2.6 cDNA Synthesis Procedure

2.2.9 Quantitative PCR

qPCR was performed using the QuantiTect SYBR Green detection kit (Qiagen), according to the manufacturer's instructions. 10 μL of QuantiTect was added to 1 μL cDNA along with 0.4 μM of forward and reverse primers, in a total reaction volume of 15 μL. Each sample was prepared in triplicate, and PCR was performed using the Roche LightCycler 480 qPCR machine. Relative amounts of mRNA were checked by comparison to the Ct value of the reference gene cyclophilin A using the Pfaffl method [236]. The cycling protocol is shown in Table 2.7 and primer sequences in Table 2.8.

Step	Purpose	Temperature (°C)	Time (min)	Cycles
1	Taq Activation	95	15	1
2	Denaturation	95	1	45
	Primer Annealing	55	0.5	
	Elongation	72	0.5	
3	Melt Curve Analysis	60	0.5	1

TABLE 2.7 qPCR Cycling Procedure

Target Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Cyclophilin A	gctttgggtccaggaatgg	gttgtccacagtcagcaatggt
NAMPT	gccagcagggaattttgtta	tgtcaccttgccattcttga
TNFα	cagagggcctgtacctcatc	ggaagacccctcccagatag
CD16	gggggcaagcatcctgggaaa	gtgggtgggtgtgccccctt
IL-8	aaaagccaccggagcactccat	agagccacggccagcttgga
IL-1β	gggcctcaaggaaaagaatc	aagtggtagcaggaggctga
ICAM1	aggggtgcatgtgttctagg	ctgacaagttgtgggggagt

TABLE 2.8 qPCR Primer Sequences

2.2.10 Whole transcriptome sequencing

RNA was isolated as described in 2.2.8 from at least 3 x 10^7 neutrophils and the RNA integrity was confirmed using the Agilent 2100 Bioanalyzer system. The total RNA concentration at this stage was routinely > 1 μg and

the RIN (RNA integrity number) was routinely >8. Following this, samples were prepared and sequenced externally, briefly, samples were enriched for mRNA using poly-A selection, and standard Illumina protocols were used to generate 50 bp read libraries, that were sequenced using the Illumina HiSeq 2000 Analyser sequencing platform at the Beijing Genomics Institute (BGI). Bioinformatic analysis was carried out in-house by H. Wright and H. Thomas, briefly, raw data was mapped to the human genome using TopHat (v1.4.1) [237] and gene annotation and calculation of RPKM expression values (the number of reads assigned to the transcript divided by the transcript length) was carried out using Cufflinks (v1.3.0) [238], with differential expression analysis carried out using the Cuffdiff tool [239]. The following analyses were carried out by the author; functional analyses were generated by Ingenuity Pathway Analysis (IPA) [240]. Canonical pathways from the IPA Knowledge Base were identified as regulated by treatment if molecules participating in the pathway set were changed < 1.5 fold from the comparison data set. The significance of the association between the data set and the identified pathway was expressed as the ratio of the number of molecules in the data set present in the pathway, divided by the total number of molecules in the pathway, and Fisher's exact test was used to calculate the probability that the association between the data set and the canonical pathway was significant.

2.2.11 Preparation of Immune Complexes

Synthetic immune complexes were prepared using human serum albumin (HSA) and anti-HSA antibody (dialysed in PBS to remove sodium azide). The concentrations of each were determined using the BCA protein assay (Qiagen), and both were then adjusted to a concentration of 5 mg/mL with PBS. 500 µg of anti-HSA antibody was added to 4 wells of a 96-well microtitre plate, and to each well a different concentration of HSA was added (10, 30, 40 or 50 µg) and made up to 200 µL with PBS. This plate was incubated for 1 h at 37°C with agitation, before determining the absorbance at 450 nm using a Bio-Rad 3550 microplate reader. The highest absorbance value obtained represented the point of equivalence where insoluble immune complexes (IIC) formed, and soluble immune complexes (SIC) formed at an antigen concentration six-times that of IIC. Larger volumes of IIC and SIC were then prepared by incubating the appropriate concentration of HSA and antibody determined by this method. Following this, the IIC were washed 3 times in PBS and any contaminating IIC in the SIC preparation was removed by centrifugation and aspiration of the SIC 3 times. The immune complexes were then stored at 4°C for up to 1 month.

2.2.12 Measurement of neutrophil ROS production

Total production of ROS (primary and secondary oxidants) from neutrophils was measured by luminol-dependent chemiluminescence. 10

μM luminol was added to 1 x 10^6 neutrophils in HBSS (37°C). The respiratory burst was induced in a receptor-independent manner, by addition of the phorbol-ester PMA (0.1 μg/mL), and measured as photon emission using a LKB 1251 tube luminometer over 40 min. The respiratory burst was also stimulated in a receptor-dependent manner using fMLP or immune complexes. Neutrophils (5 x 10^6/mL) were primed for 30 min with GM-CSF (50 U/mL), then 10 μM luminol was added to 2 x 10^5 cells and the respiratory burst was stimulated with 1 μM fMLP or 20% (v/v) immune complexes, with HBSS in a total volume of 200 μL in a 96-well white, opaque microtitre plate. The oxidants produced over 40 min were measured using a Perkin Elmer Victor plate reader. The rate of production of solely extracellular reactive oxidants, produced at the cell membrane, was assayed in a similar manner, using 1 μM isoluminol in place of luminol. The signal produced with isoluminol is weaker, so it was enhanced by addition of HRP (4 U/mL). In this case, the respiratory burst was stimulated with 1 μM fMLP, and the oxidants produced over 10 min were assayed using the FLUOstar Omega plate reader from BMG Labtech.

The rate of extracellular production of O_2^- via the neutrophil NADPH oxidase, can be measured by O_2^- mediated reduction of ferri-cytochrome c (λ_{max} 550 nm). 75 μM ferri-cytochrome c was added to 5 x 10^6 neutrophils in a total volume of 1 mL HBSS (37°C), and PMA (0.1 μg/mL) was added to stimulate the respiratory burst. Reduction of ferri-cytochrome c was

measured spetrometrically at 550 nm using the Perkin-Elmer Lambda 5 spectrophotometer, as compared to a reference cuvette with no PMA. Absorbance at 550 nm was recorded at 1 min intervals over 10 min. The rate of O_2^- secretion was calculated, using the millimolar extinction coefficient of cytochrome c (19.1), and expressed as nM/min/10^6 cells.

2.2.13 Preparation of S. aureus

10 mL of sterile LB broth was inoculated with a single *S. aureus* colony (from an LB agar streak plate) and incubated overnight at 37°C with agitation. A subculture was then prepared from this overnight culture and grown to mid-exponential growth phase. At this point, the culture was serially diluted, the absorbance was recorded at 540 nm, and viable counts were taken by plating onto LB agar plates. A calibration curve was then constructed so that the number of bacterial cells could be determined spectrometrically at 540 nm. For each experiment, an overnight culture was prepared from a streak plate as above, and the cells were adjusted to a concentration of 1 x 10^{10} cells/mL in LB broth.

2.2.14 Assessment of neutrophil killing capacity

For assay of neutrophil killing capacity, live *S. aureus* were prepared as described above, and then opsonised with 30% (v/v) human AB serum in PBS at 37°C with agitation. After 20 min, the cells were washed twice in PBS to remove the serum. Alongside this, freshly isolated neutrophils were

incubated for 30 min with media alone or 100 nM FK866 at 37°C with agitation. The opsonised bacteria were then added to the neutrophils at a ratio of 10:1 and incubated for 1 h at 37°C with gentle agitation. Following this, the neutrophils were lysed in ddH_2O, and the remaining bacteria were serially diluted in PBS before plating onto LB agar plates at a concentration yielding 30-300 colonies. The plates were incubated overnight at 37°C, and the killing capacity of the neutrophils was assessed by comparing the number surviving bacteria (cfu) not killed by the neutrophils in culture. Cultures without neutrophils were prepared alongside to determine the total number of bacteria.

2.2.15 Assessment of neutrophil phagocytosis and respiratory burst

For assay of neutrophil phagocytosis, *S. aureus* were prepared as described above, then 1 x 10^{10} bacteria were heat killed by incubating at 60°C for 30 min. Bacteria were then washed and resuspended in 1 mL PBS with 30 µM PI, and incubated in the dark at 4°C for 2 h. The bacteria were then washed 3 times in HBSS (0.1% gelatine), prior to opsonisation with 30% human AB serum for 1 h at 37°C with agitation. Following this, bacteria were washed and resuspended in PBS at a concentration of 1 x 10^{10} cells/mL, and stored at 4°C for up to 1 month. Freshly isolated neutrophils (1 x 10^6/mL) were incubated in media alone (control) or in the presence of 100 nM FK866 for 30 minutes at 37°C with agitation, before bacteria were added at a ratio of 10:1 bacteria to neutrophils. The cells were incubated

for 15 min, before 5 μM DHR123 was added for a further 15 min, alongside the appropriate untreated controls. Following this, the cells were resuspended in 2 mL HBSS, and a 200 μL aliquot was added to a 96-well microtitre plate for flow cytometry.

2.2.16 Inhibition of NAMPT with FK866

The NAMPT inhibitor FK866 was reconstituted in DMSO, preliminary experiments using DMSO alone confirmed it had no effect on the neutrophil functions tested. NAMPT was inhibited using a range of 1-1000 nM FK866, with 100 nM used in the majority of the experiments. The IC_{50} of FK866 is 1 nM, however some studies have identified that this dose is ineffective in certain cell lines [241]. FK866 was developed as an anti-cancer agent that causes a gradual depletion in cellular NAD content and eventually leads to cell death after a number of days [206]. However, neutrophils are relatively short-lived cells, and the functional capacity of these cells quickly declines in culture; this necessitated the length of incubation for most functional assays performed in this thesis to be 1 hour or less. NAMPT inhibition had the greatest effect when time was allowed for NAD(P)H depletion to occur prior to stimulation of function. Where possible, neutrophils were pre-treated for 1 h, however for many functional tests that relied on membrane receptor expression, control neutrophils became less responsive after a >30 min pre-incubation time. The limited incubation time, and early experiments demonstrating the effect of FK866

on neutrophil NAD content and the respiratory burst led to the conclusion that FK866 would be used at a range of 50-100 nM.

2.2.17 Statistical analysis

Data was judged for significance using a combination of parametric and non-parametric statistical tests. Paired data was analysed using the parametric Student's t-test, making the assumption that the data was normally distributed. When comparing non-normally distributed data that were matched but not necessarily from the same sample or analysed at the same time, a non-parametric test such as the Wilcoxon signed-rank test was used (as indicated in the text). Data are displayed ± SEM unless otherwise stated, and differences were considered significant if $p \leq 0.05$.

CHAPTER 3: Effects of NAMPT Inhibition on Neutrophil Function

3.1 Introduction

In addition to the ability of exogenous NAMPT to regulate neutrophil functions, such as priming and apoptosis [19,224], this molecule is also expressed by neutrophils [19]. Intracellularly, NAMPT catalyzes the conversion of nicotinamide (NAM) to nicotinamide monophosphate (NMN). This is the rate-limiting step of NAD biosynthesis in the NAD salvage pathway [23,26]. By controlling NAD recycling, NAMPT can impact cellular energetics and also the activity of NAD-dependent enzymes, such as the NADPH oxidase of phagocytes, assembly and activation of which represents the first stages of the neutrophil respiratory burst.

The enzymatic activity of NAMPT can be blocked by the inhibitor FK866. Inhibition of NAMPT significantly decreases secretion of TNFα, IL-1β and IL-6 from inflammatory cells *in vitro*, while *in vivo* this inhibition decreases serum TNFα and the severity of mouse models of inflammatory arthritis [33,34]. The ability of NAMPT to stimulate expression of cytokines, such as IL-1β, and destructive enzymes, such as MMPs, coupled with its role in mediating neutrophil apoptosis, identify this protein as a potentially important mediator of inflammatory disease.

In view of the anti-inflammatory potential of NAMPT inhibitors and the role of neutrophils in the pathogenesis of inflammatory diseases, it is important to define the role of NAMPT in neutrophil functions. Inhibition of NAMPT activity in neutrophils could decrease their pro-inflammatory activity, but could also impair their ability to combat bacterial infections. The latter could have severe consequences on morbidity and mortality of patients treated with NAMPT inhibitors.

3.2 Aims

The aims of this chapter were to determine the effects of NAMPT inhibition on NAD(P)H levels in neutrophils, and how this might affect neutrophil activities relevant in inflammation. It was hypothesized that inhibition of NAMPT, and thus the NAD salvage pathway, would decrease the availability of NAD/H and possibly NADP/H, which may affect redox reactions and the action of NAD(P) consuming enzymes, including the NADPH oxidase. Therefore, a variety of neutrophil functions were tested following NAMPT inhibition, using FK866 alone, and in combination with priming agents such as GM-CSF and TNFα. These experiments would determine if inhibition of this pathway could disrupt inflammatory processes, and provide the first experiments investigating the role of this enzyme in neutrophil function.

3.3 Results

3.3.1 NAMPT inhibition depletes intracellular NAD(P)H

NAMPT is reported to play a key role in the salvage pathway by catalyzing the production of NMN from nicotinamide and PRPP [26]. Therefore, inhibition of NAMPT by FK866 may be predicted to decrease intracellular NAD levels and this may affect neutrophil viability and functions that directly or indirectly depend on this molecule. Cellular NAD(P)H content was initially measured using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay or the MTS assay (Promega). MTS is a tetrazolium salt that can be reduced to a water soluble fomazan product by intracellular dehydrogenases in the presence of NADH and NADPH. The rate of formazan production is directly related to the production of NADH and NADPH, and can be assayed at A_{490} [242]. Control neutrophils were incubated for 4 h with increasing concentrations of the NAMPT inhibitor, FK866, and then a further 2 h with the MTS reagent. Relative NAD(P)H content was assayed at A_{490}, and cell viability was measured alongside using the Viacount assay. Under these experimental conditions, FK866 triggered a dose-dependent decrease in intracellular NAD(P)H, by up to 40% at the highest concentration tested (1000 nM, $p<0.01$), but had no effect on the viability of the neutrophils, as determined by the Viacount assay. A significant (25%, $p<0.05$)

decrease in intracellular NAD(P)H was detected at concentrations of FK866 as low as 10 nM (Fig. 3.1A).

The decrease in intracellular NAD(P)H with FK866 was confirmed in another assay, using the Amplite™ Colormetric NAD/H or NADP/H assay kit (AAT Bioquest), which allows the absolute concentration of NAD/H and NADP/H to be determined by comparison to a standard curve. 5 x 10^6 cells were incubated at 37°C either with media alone, GM-CSF (50 U/mL), or FK866 (50 or 100 nM), for the indicated time periods. Cells were also pre-treated for 1 h with the inhibitor or media alone prior to stimulation with either GM-CSF or PMA (0.1 μg/mL) for a further 1 h. Figure 3.1B shows that the concentration of NAD/H increased over time in culture in untreated cells. This increase was abrogated by NAMPT inhibition; the NAD/H concentration was significantly lower than in resting cells after 2 h incubation with 50 nM FK866 (41% $p<0.05$) and after 1 h with 100 nM FK866 (19% $p<0.05$). NADP/H concentration appeared to increase over time in culture (63 % over 4 h), and again FK866 at 50 nM (64% decrease at 4 h) and 100 nM (53% decrease at 4 h) abrogated this increase, but this did not reach statistical significance.

To observe the changes in intracellular NAD/H and NADP/H that occur during the typical incubation used for the following experiments, neutrophils were incubated in the absence or presence of 100 nM FK866

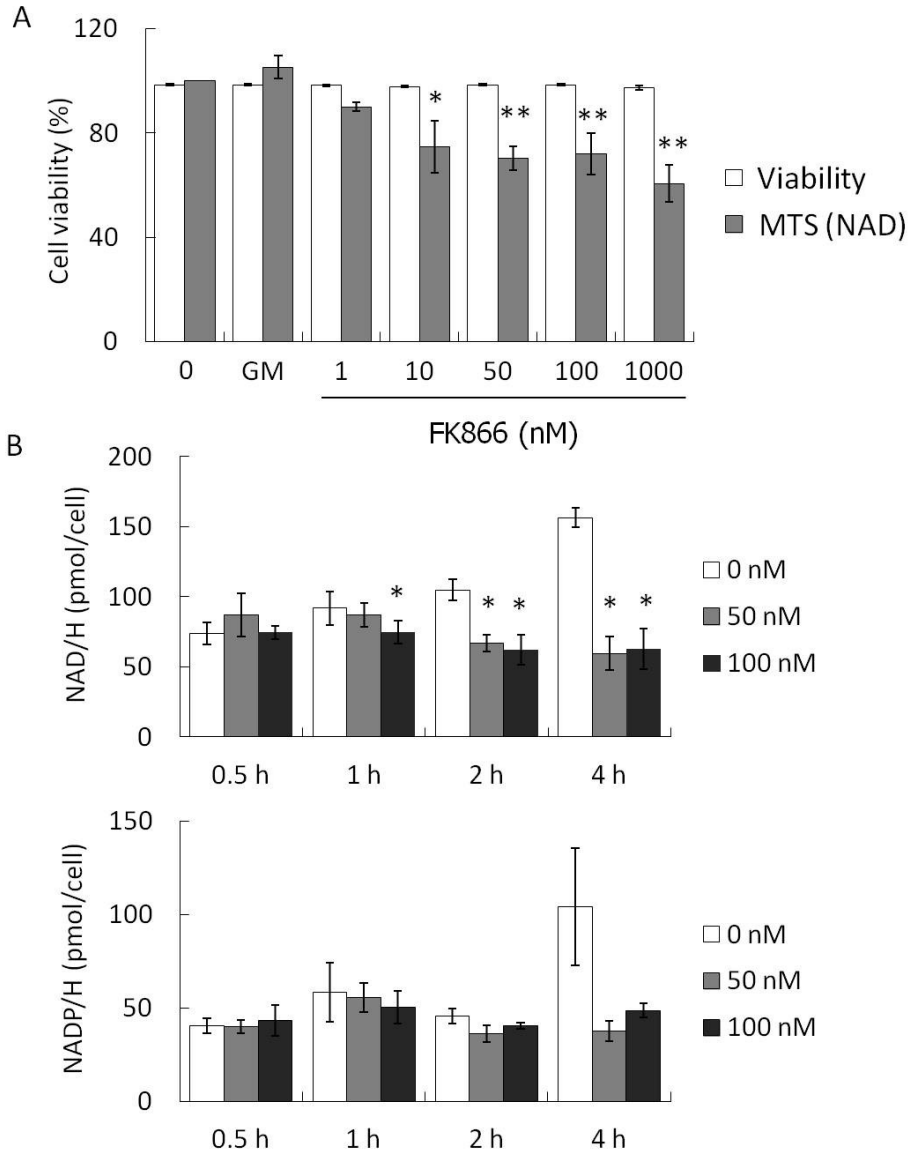

FIGURE 3.1: NAMPT inhibition with FK866 depletes resting neutrophil intracellular NAD(P)/H. **A.** Cellular NAD content was assayed indirectly at 4 h by MTS assay and compared to cell viability by the Viacount flow cytometric assay. Data represent mean ±SEM of 5 independent experiments. GM - GM-CSF **B**. Neutrophil NAD/H and NADP/H contents were measured alternatively using a colorimetric assay at the indicated timepoints with either 50 or 100 nM FK866 as indicated, data represent mean ±SEM of 5 independent experiments, and statistics are calculated as change from 0 nM. *$p<0.05$, **$p<0.01$.

for 1 h and then stimulated with either GM-CSF or PMA for a further 1 h or 5 min respectively. Both PMA and GM-CSF induced a modest increase in NAD/H (3.2A,B), however, PMA induced a much larger increase in NADP/H (3.2C,D), likely due to stimulation of the respiratory burst via the NADPH oxidase. Pre-treatment with the NAMPT inhibitor prior to stimulation, abated the upregulation of both NAD/H and NADP/H in response to these stimulants (Fig. 3.2).

Because many neutrophil functions directly or indirectly require NAD, it was then necessary to determine the effects of NAMPT inhibition on the properties of these cells.

3.3.2 Effects of NAMPT inhibition on the neutrophil respiratory burst

The neutrophil respiratory burst that results in the generation of a series of reactive oxygen species (ROS) is triggered via activation of the NADPH oxidase to initially generate O_2^- [243]. As the respiratory burst requires electrons transferred from NADPH to O_2, it was necessary to determine if inhibition of NAMPT and disruption of the NAD salvage pathway, would negatively affect the activity of this NADPH oxidase and hence the ability of neutrophils to generate ROS.

Neutrophils were incubated for 1 h in the absence and presence of increasing concentrations of FK866, and the respiratory burst, triggered by the addition of the phorbol ester, PMA, was measured by luminol-

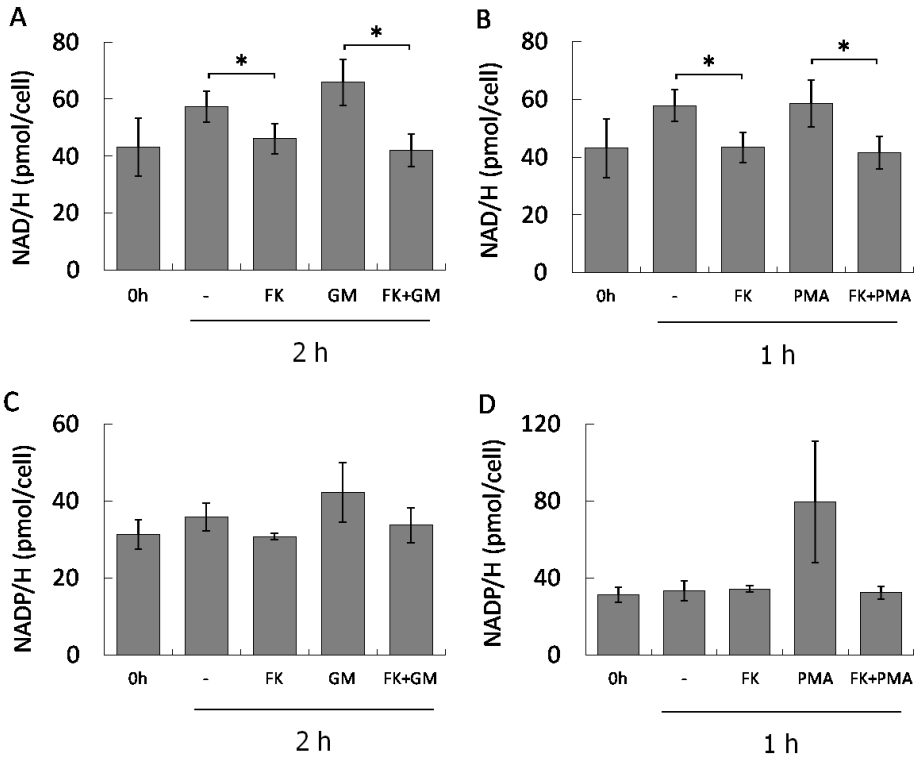

FIGURE 3.2: NAMPT inhibition with FK866 decreases neutrophil intracellular NAD(P)/H in response to priming and stimulation. Neutrophil NAD/H and NADP/H content was measured using a colorimetric assay. Neutrophils were pre-incubated with 100 nM FK866 as indicated (FK) for 1 h, then for either a further 1 h with GM-CSF (50 U/mL) (GM), or a further 5 min with PMA (0.1 µg/mL). Data represent mean ±SEM of 4 independent experiments, *$p<0.05$.

dependent chemiluminescence. Fig. 3.3A shows that under these conditions, FK866 resulted in a dose-dependent inhibition of the respiratory burst, with over 50% inhibition observed at a concentration of the inhibitor of 100 nM. As well as being concentration-dependent, this inhibition of the respiratory burst was also time-dependent (Fig. 3.3B): at a concentration of 10 nM FK866, inhibition of the respiratory burst was significant by 30 min pre-incubation with the inhibitor, and this inhibitory effect was greater as the pre-incubation period increased. Representative traces of this inhibition of respiratory burst activity are shown in Fig. 3.3C, and this inhibitory effect of FK866 was also observed when NADPH oxidase activity was measured as the rate of O_2^- secretion using the cytochrome c reduction assay (Fig. 3.3D).

PMA activates the neutrophil respiratory burst by directly activating protein kinase C via a mechanism that is independent of receptor-coupled signalling [244]. However, neutrophil function is usually activated *in vivo* by receptor-linked signalling [48]. Therefore, the effects of FK866 on activation of the respiratory burst triggered by a number of ligands of patho-physiological importance were also determined.

Neutrophil activation *in vivo* is usually regulated by prior exposure to cytokines, such as GM-CSF, that can prime the cells into a state of enhanced responsiveness [3]. The effects of NAMPT inhibition on

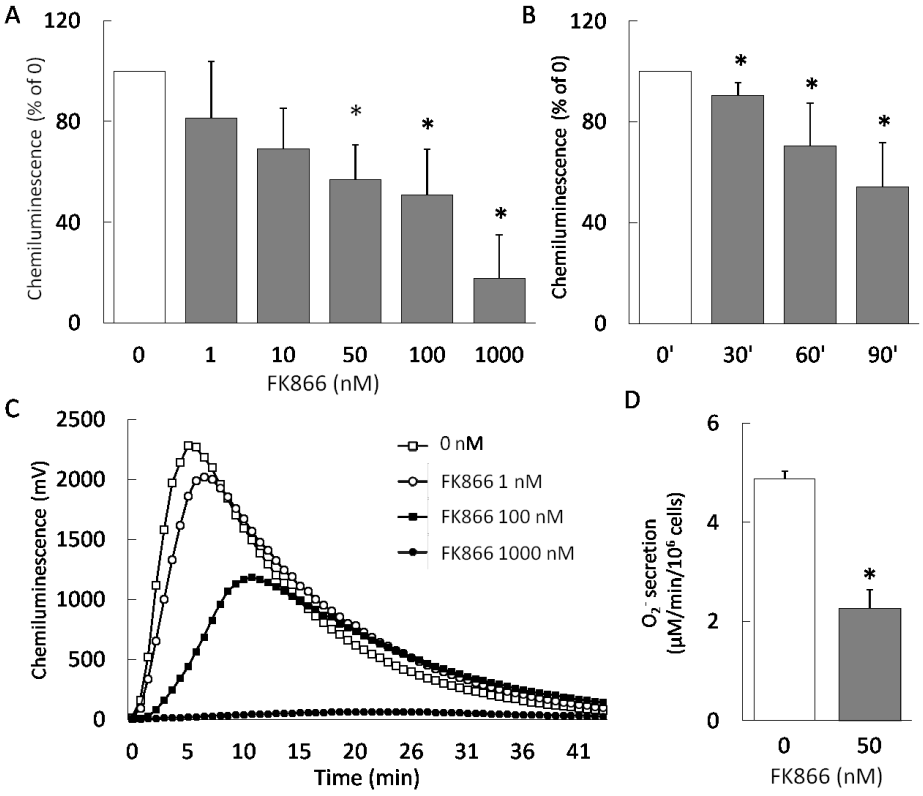

FIGURE 3.3: FK866 inhibits the neutrophil respiratory burst in a time- and dose- dependent manner in response to PMA. Neutrophils were incubated for 60 min with the indicated concentration of FK866 (**A**) or for the indicated time with 10 mM FK866 (**B**) total ROS were measured by luminol-enhanced chemiluminescence in response to PMA (0.1 µg/mL). Data represent mean ±SEM of 5 independent experiments, representative trace shown in **C**. **D**, the rate of neutrophil extracellular release of O_2^- was measured spectrophotometrically by O_2^- mediated reduction of ferri-cytochrome c (λ_{max} 550 nm). Data represent mean ±SEM of 3 independent experiments, *$p<0.05$.

unprimed and GM-CSF primed neutrophils were also determined in these experiments, to assess if NAMPT was involved the priming response.

In these experiments neutrophils were incubated for 30 min in the absence or presence of 100 nM FK866 and then for a further 30 min in the absence or presence of GM-CSF. Unprimed neutrophils generated only low levels of reactive oxidants when stimulated by fMLP, but this low level of ROS production was significantly decreased ($p<0.05$, $n=8$) in the presence of the NAMPT inhibitor. Priming by GM-CSF significantly increased ROS production stimulated by fMLP, and this primed response was also inhibited by FK866 (Fig. 3.4A). In inflammatory disorders such as rheumatoid arthritis, primed neutrophils can be inappropriately stimulated to produce ROS in response to soluble and insoluble immune complexes [163]. We therefore determined the effects of priming and NAMPT inhibition on activation of ROS production triggered by immune complexes. Previous work [161,245] has shown that soluble and insoluble immune complexes can activate neutrophils by different signalling pathways. Both the unprimed and GM-CSF primed ROS production, triggered by either type of immune complex, were similarly decreased in the presence of FK866 (Fig. 3.4B,C).

Neutrophils produce ROS both inside the phagosome and at the cell surface, neutrophils can also be stimulated to secrete ROS; bystander tissue injury can occur when ROS are released extracellularly. To

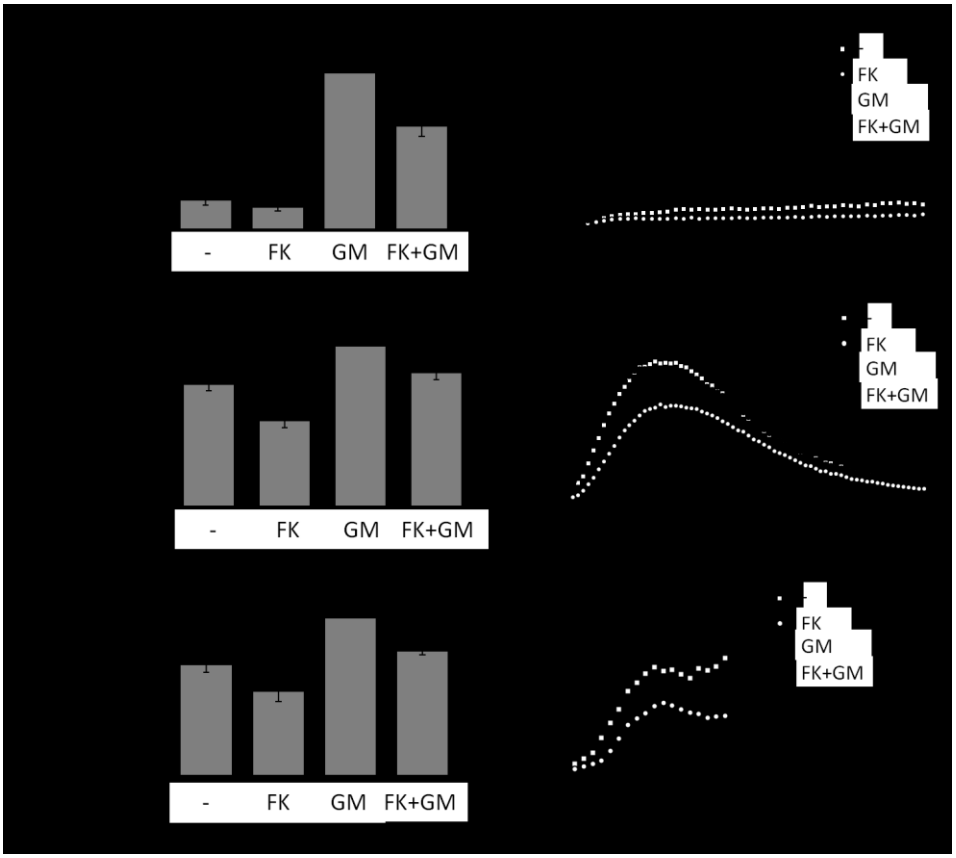

FIGURE 3.4: FK866 inhibits the neutrophil respiratory burst in response to fMLP and immune complexes, in both primed and unprimed cells. Neutrophils were pre-incubated in the absence (-) and presence (FK) of 100 nM FK866 for 30 min, then incubated for a further 30 min in the absence or presence of GM-CSF (GM). The respiratory burst was then stimulated with either 1 μM fMLP (**A**), or 20% insoluble (IIC) (**B**), or soluble immune complexes (SIC) (**C**), and measured by luminol-enhanced chemiluminescence. Data (left panel) represent mean ±SEM of 8 independent experiments, and representative traces are shown in the right panel, *$p<0.05$, ***$p<0.001$.

determine whether the inhibition of ROS affects both intra- and extracellular ROS production, ROS were measured using the membrane impermeable isoluminol alongside the membrane permeable luminol. Neutrophils were pre-incubated as previously in the absence or presence of 100 nM FK866 for 30 min and then incubated for a further 30 min in the presence and absence of GM-CSF. The respiratory burst was then stimulated with either fMLP (1μM) or PMA (0.1μg/mL) and measured using either luminol- or isoluminol-enhanced chemiluminescence. The level of ROS produced extracellularly is below the level of detection with isoluminol alone, so HRP was included to enhance the fluorescence. Extracellular ROS were decreased in an equal manner to total ROS produced, and it can be seen that FK866 appears to have a greater inhibitory effect on receptor-mediated production of ROS, than that stimulated synthetically (Fig. 3.5).

3.3.3 Effect of FK866 on the expression of neutrophil cell surface receptors

Immune complexes activate the neutrophil respiratory burst via Fcγ receptors [162,245]. The expression of the major Fcγ receptor on neutrophils, CD16b (FcγIIIb), is dynamically regulated by its rate of shedding, its rate of mobilization to the plasma membrane from intracellular stores and possibly by its rate of biosynthesis [64,246,247]. We therefore measured the effects of NAMPT inhibition on the regulation of this receptor in both

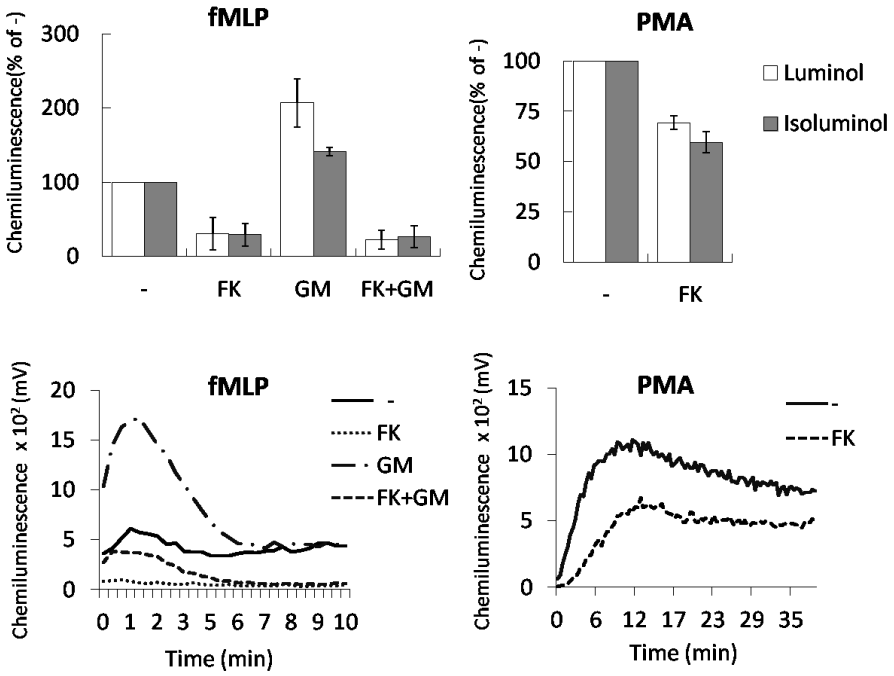

FIGURE 3.5: FK866 inhibits the production of both intracellular or extracelluar ROS. Neutrophils were pre-incubated in the absence (-) and presence (FK) of 100 nM FK866 for 30 min, then incubated for a further 30 min in the absence or presence of GM-CSF (GM). The respiratory burst was then stimulated with either 1 μM fMLP or 0.1 μg/mL PMA, and measured by either luminol- or isoluminol-enhanced chemiluminescence. Data represent mean ±SD of 2 independent experiments and representative isoluminol traces are shown.

control and GM-CSF treated cells, this was firstly assessed by immunoblotting for CD16b shed from the cell surface into the culture supernatant. No significant changes in cellular expression of CD16b were observed (Fig. 3.6A). The cell culture supernatants were probed for CD16b cleaved from the surface of the cell, and there was a significant increase in CD16b shedding following GM-CSF treatment (Fig. 3.6A). However, NAMPT inhibition, prior to stimulation, abrogated the GM-CSF induced shedding (Fig. 3.6A).

Flow cytometry was then used to determine the levels of cell surface and intracellular CD16b following NAPMT inhibition. Neutrophils were pre-incubated for 1 h with FK866 (100 nM), then a further 1 h with GM-CSF, alongside the appropriate no treatment controls. Incubation of neutrophils with 100 nM FK866 alone resulted in very little change in the surface expression of CD16b, but a statistically-significant decrease in the total cellular level of this receptor, due to a significant decrease in the intracellular receptor pool (Fig. 3.6B): a similar effect was seen with GM-CSF treatment. Previous work [64] has shown that GM-CSF results in an increase in shedding of CD16b from the cell surface and a concurrent mobilization of the receptor from internal stores, maintaining cell surface expression. This was confirmed in Figure 3.6B where a statistically-significant decrease in the intracellular pool of CD16b was observed, but the surface expression remained unchanged from control. This effect was

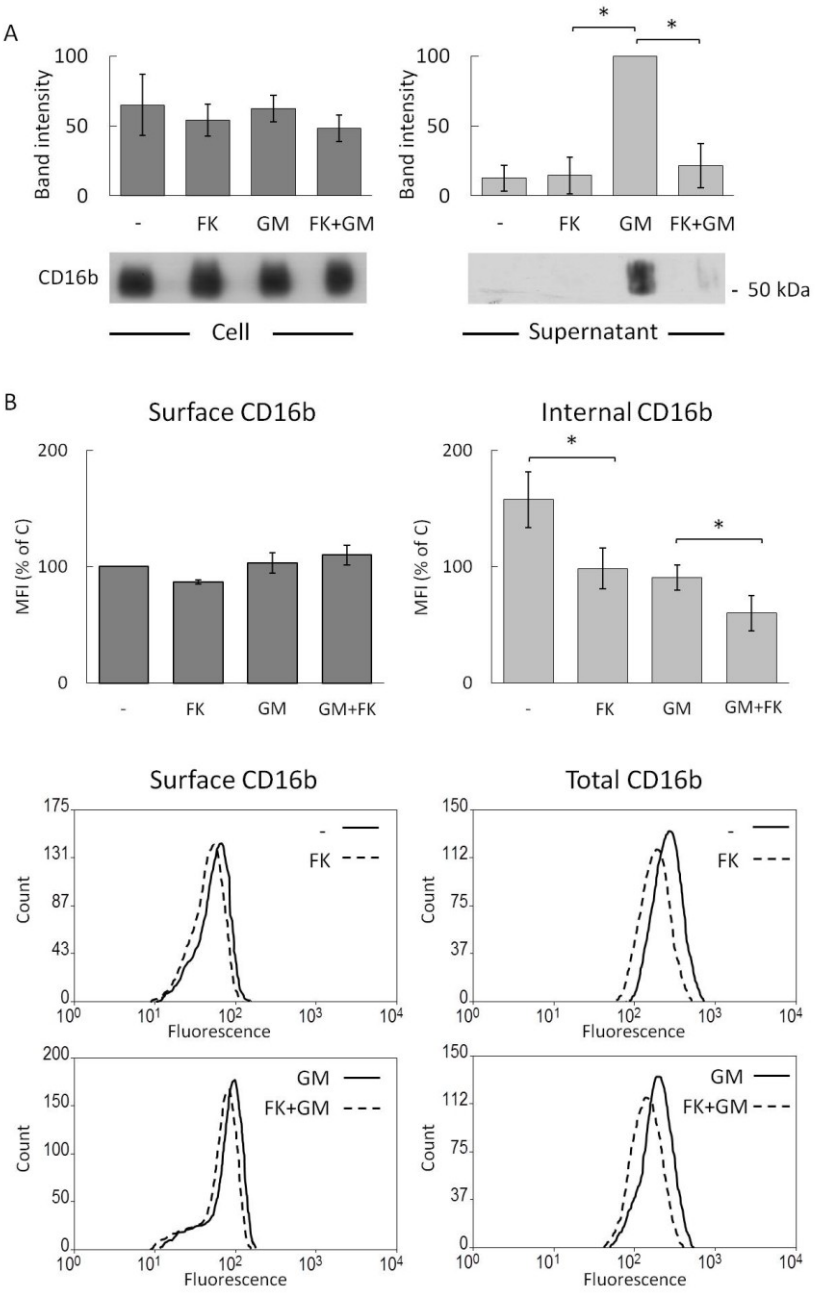

FIGURE 3.6: FK866 inhibits GM-CSF induced shedding of CD16b, and decreases intracellular pools, but does not significantly affect its expression on the neutrophil surface. A. Neutrophils were incubated for 1 h with 100 nM FK866 (FK) or medium alone (-), then a for further 1 h in the presence and absence of GM-CSF (GM), total cellular CD16b and CD16b shed into the culture supernatant were then assessed by immunoblotting. Densitometry represents the mean ±SEM of 3 independent experiments and representative blots are shown below. **B**. Surface expression and intracellular stores of CD16b were measured by flow cytometry of neutrophils treated in the same way, then fixed and permeabilized. Data represent mean ±SEM of 5 independent experiments and representative traces are shown below, *p<0.05.

compounded when cells were treated with GM-CSF and FK866 in combination, and there was a further decrease in intracellular CD16b compared to GM-CSF treatment alone (Fig. 3.6B). Thus, NAMPT inhibition affects the dynamics of CD16b synthesis and shedding in neutrophils, but cell surface expression is maintained, and as such is unlikely to have an adverse effect on receptor mediated events, such as activation of the respiratory burst.

NAMPT inhibition (1 h, 100nM) had no effect on the surface expression of other key neutrophil surface markers tested in the presence and absence of GM-CSF priming. CD11b on the surface of neutrophils is normally up-regulated by GM-CSF [48,50,60,248], and this cytokine-dependent up-regulation was not affected by NAMPT inhibition (Fig. 3.7A). Similarly, NAMPT inhibition alone did not alter CD11b expression in non-cytokine treated cells. In contrast to the cytokine-dependent up-regulation of CD11b expression, GM-CSF triggers the shedding of L-selectin (Fig. 3.7B). Again, NAMPT inhibition by FK866 did not affect either the expression of L-selectin on resting neutrophils, or the cytokine-induced shedding of this receptor. Similarly, neither the resting levels of expression of the Fcγ receptor, CD32, nor the levels of expression of this receptor observed after GM-CSF treatment, were affected by NAMPT inhibition (Fig. 3.7C). Therefore, under these

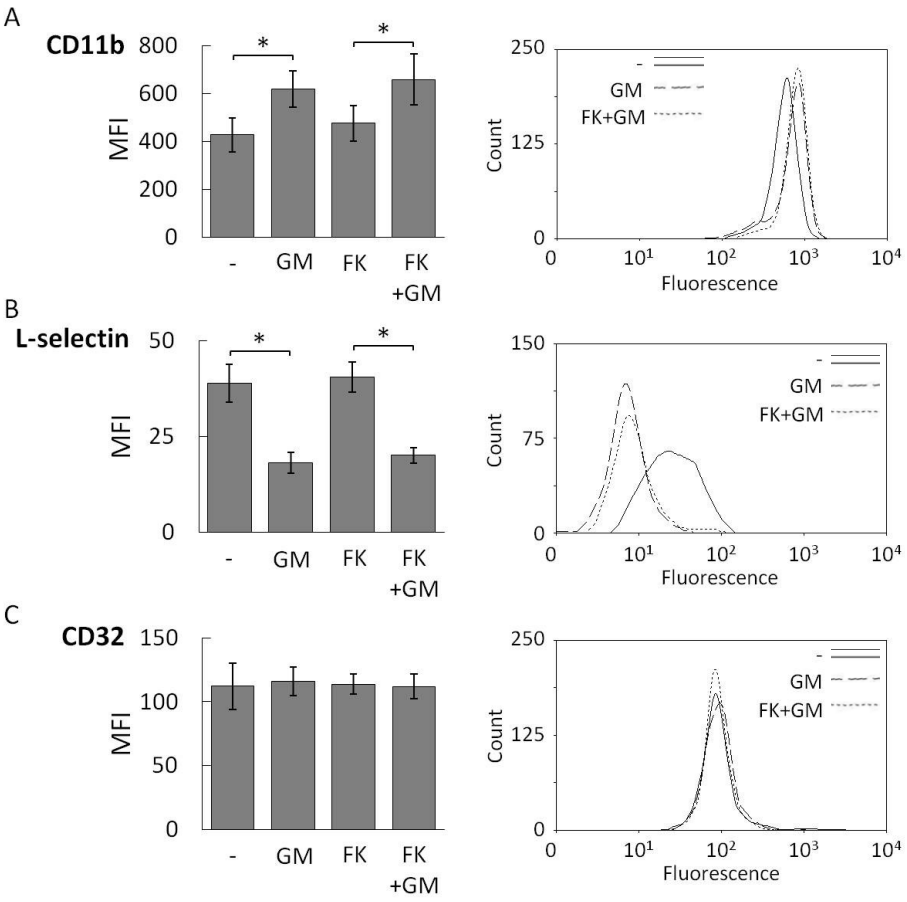

FIGURE 3.7: FK866 does not affect the surface expression of other key neutrophil surface receptors. Neutrophils were incubated for 1 h with 100 nM FK866 or media alone (-), then a further 1 h in the presence and absence of GM-CSF (GM), and surface expression of CD11b (**A**), L-selectin (**B**) and CD32 (**C**) was measured by flow cytometry. Data represent mean ±SEM of 4 independent experiments (left panel) and representative traces are shown on the right, *$p<0.05$

experimental conditions, NAMPT inhibition did not affect the level of cell surface expression of any of these important neutrophil receptors.

3.3.4 Effect of NAMPT inhibition on phagocytosis and degranulation

Having shown that NAMPT inhibition decreased the ability of neutrophils to generate ROS production in response to soluble agonists, it was then necessary to determine if it also affected ROS production during phagocytosis of bacteria. In the first sets of experiments, the rates of phagocytosis of PI-stained (heat-killed) serum-opsonised *S. aureus* were measured. FK866 did not affect phagocytosis of these serum-opsonised bacteria (Fig. 3.8A), in line with its lack of effect on the surface expression of receptors likely to be involved in opsono-phagocytosis. The rate of intraphagosomal ROS production during phagocytosis of *S. aureus*, was then measured with the same FK866 pre-treatment, using Dihydrorhodamine (DHR) 123 and flow cytometry. DHR123 is a cell-permeable dye that becomes fluorescent once oxidised by ROS [249]. In contrast to its lack of effect on phagocytosis, and in agreement with the effects observed previously, the NAMPT inhibitor significantly decreased intraphagosomal ROS production (Fig. 3.8A). Under these experimental conditions (100 nM FK866; 30 min pre-incubation), intraphagosomal ROS production was decreased by approximately 65% ($p<0.01$, $n=5$).

Following phagocytosis, neutrophils release granule enzymes, alongside ROS. To investigate whether NAMPT inhibition affects this process, the

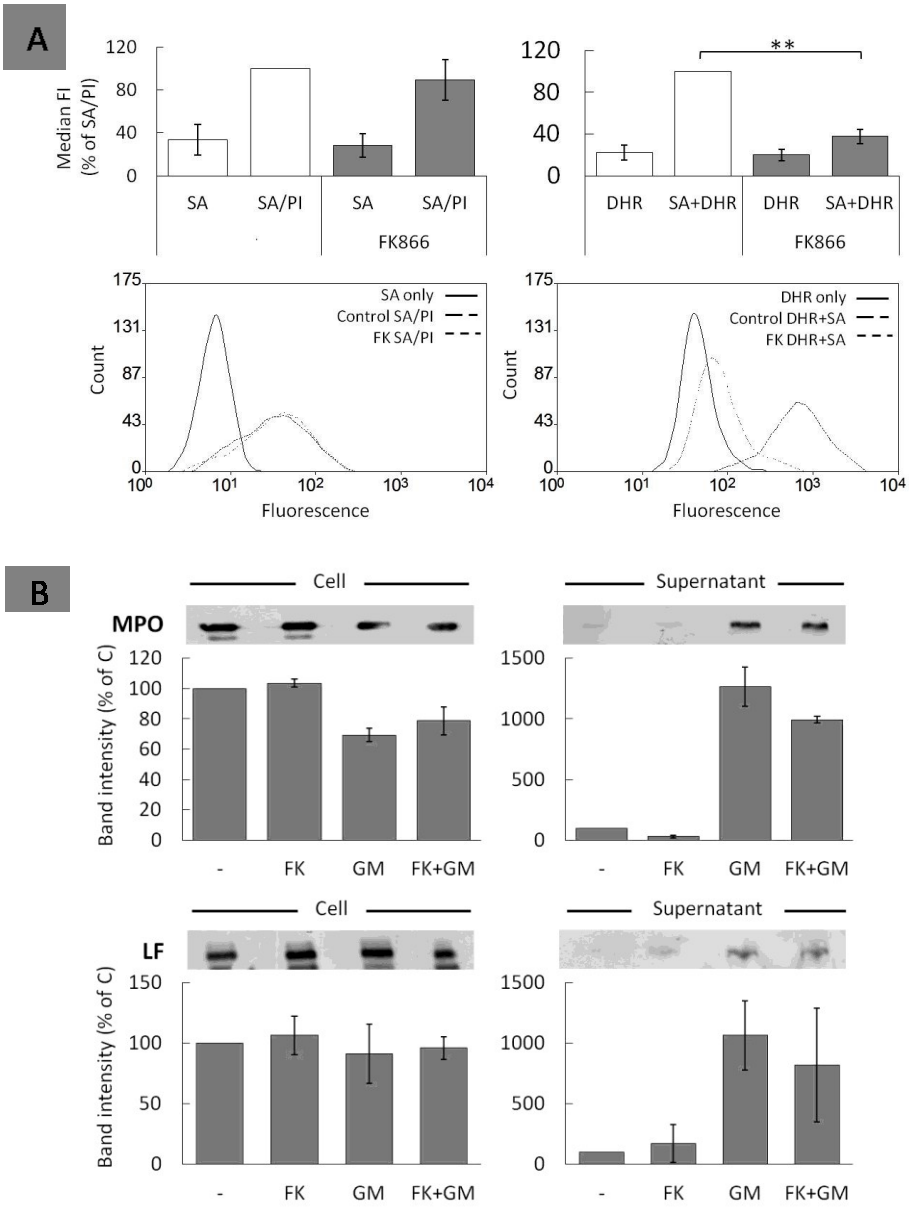

FIGURE 3.8: FK866 inhibits production of ROS in response to *S. aureus,* but does not affect phagocytosis or release of granule proteins. A. Neutrophils were pre-incubated with 100 nM FK866 (FK) or media alone (-) for 30 min, then with opsonised *S. aureus* (SA) either unlabelled or labelled with PI (SA/PI) for 30 min; uptake was measured by flow cytometry. **B**. Alternatively, following 15 min incubation with *S. aureus*, or media alone, DHR123 was added to samples for 15 min to measure intraphagosomal ROS production. Data represent the mean ±SEM of 5 experiments with representative traces. **C.** Neutrophils were pre-incubated for 1 h with 100 nM Fk866, then 30 min in the presence and absence of GM-CSF (GM). All samples were then treated with cytochalasin B for 10 min and 1 μM fMLP for a further 10 min to stimulate degranulation. Total cellular myeloperoxidase (MPO) and lactoferrin (LF) and that released into the culture supernatant was assayed by immunoblotting. Densitometry represents the mean ±SEM of 4 experiments with representative blots, **p<0.01.

release of two of these granule proteins, myeloperoxidase (MPO) (primary granules) and lactoferrin (LF) (secondary granules), was measured in response to fMLP, following pre-incubation with media alone or FK866. Neutrophil preparations were also treated with cytochalasin B, which disrupts the actin cytoskeleton, and allows release of the granule enzymes into the culture supernatant following stimulation. MPO and LF in the culture supernatant were then measured by immunoblotting. Pre-incubation with FK866 (100 nM for 1 h) had no significant inhibitory effect on the release of these granule enzymes (Fig. 3.8B).

3.3.5 FK866 does not affect neutrophil chemotaxis towards fMLP

It was investigated whether inhibition of NAMPT affected the ability of neutrophils to transmigrate towards fMLP. Hanging culture well inserts with a 3 μm polycarbonate filter were suspended in a culture plate containing media and the indicated chemoattractant. Following this, 1 x 10^6 neutrophils, either untreated or following 30 min with FK866, were added into the hanging well inserts, and incubated at 37°C for 1 h. The cells that had migrated through the filter towards the chemoattractant were counted using a Multisizer 3 cell counter. 10 nM fMLP was used as a positive control for chemotaxis. Around 50% ($p<0.05$) of the cells transmigrated through the membrane towards fMLP, and this was unaffected by pre-treatment with FK866 (Fig. 3.9).

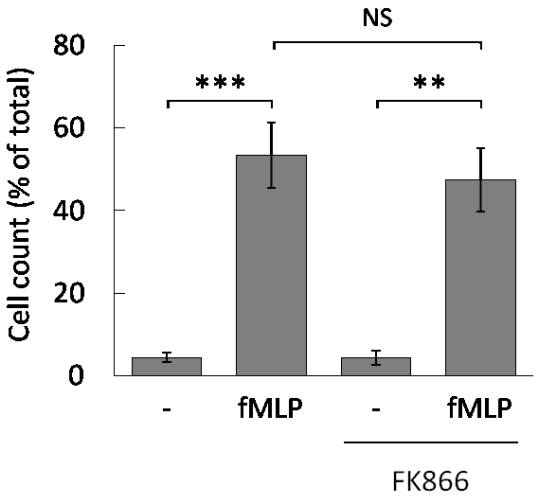

FIGURE 3.9: FK866 does not affect neutrophil chemotaxis towards fMLP. Neutrophils were incubated for 30 min with 100 nM FK866, then added into a hanging well insert with a 3 μm membrane suspended above media alone (-) or media containing 10 nM fMLP for 1 h. The neutrophils that had migrated through the membrane were then counted using a cell counter. Data represent mean ±SEM of 7 independent experiments, **p<0.01, ***p<0.001.

3.3.6 Effect of NAMPT inhibition on bacterial killing.

Activation of the neutrophil NADPH oxidase is a key event in regulating bacterial killing within the phagosome [1]. In view of the inhibitory effects of FK866 on ROS production, particularly intraphagosomal ROS production following phagocytosis of bacteria, its effects on bacterial killing were determined. Neutrophils (5×10^6) were pre-incubated for 30 min in the absence (control) or presence of 100 nM FK866 and then for a further 1 h in with serum-opsonised *S. aureus* (at a bacteria:neutrophil ratio of 10:1). Following this, the neutrophils were lysed in water, and the remaining bacteria were serially diluted and spread onto agar plates. Following incubation overnight at 37°C, the number of colony forming units (cfu) were counted, and compared to neutrophil free cultures. Under these conditions, control neutrophils killed approximately 40% of the bacteria (Fig. 3.10), but inhibition of NAMPT by FK866 had no significant effect on bacterial killing. Therefore, in spite of the large decrease in ROS production following NAMPT inhibition, this did not affect the efficiency of bacterial killing.

3.3.7 Effect of NAMPT and FK866 on neutrophil apoptosis

Previous work has shown that the addition of exogenous NAMPT to neutrophils can delay their rate of apoptosis [19]. Previous work [38] has also shown a key role for the anti-apoptotic protein, Mcl-1 in regulating the

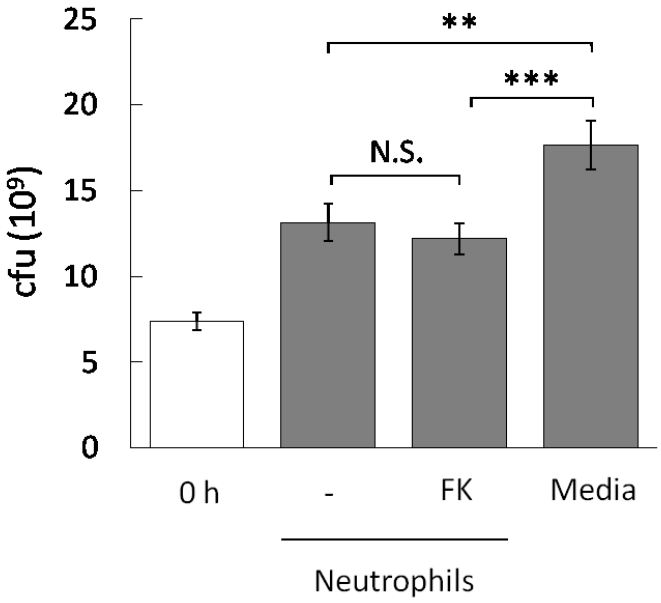

FIGURE 3.10: Inhibition of ROS production with FK866 does not have any effect on neutrophil killing of opsonised *S. aureus.* Neutrophils were pre-incubated with 100 nM FK866 or media alone (-) for 30 min, prior to mixing with opsonised *S. aureus* for 1 h. Following this neutrophils were lysed and the number of bacteria killed was assayed by comparing the cfu remaining following different neutrophil treatments and compared to a no neutrophil control (media). Data represent mean ±SEM of 4 independent experiments, **p<0.01, ***p<0.001, N.S. – not significant.

rate of neutrophil apoptosis. The addition of exogenous NAMPT to neutrophils (100 ng/mL) significantly delayed their apoptosis over a 17 h incubation period and maintained Mcl-1 protein levels at 5 h (Section 4.3.4). However, intracellular NAMPT inhibition by FK866 had no significant effect on apoptosis at 17 h (Fig. 3.11A), neither decreasing nor increasing viability. Also, NAMPT inhibition had no effect on degradation of Mcl-1; Mcl-1 was significantly decreased in control cells undergoing high rates of apoptosis by 5 h, and this was unchanged by addition of FK866 (Fig. 3.11B).

3.4 Discussion

NAMPT functions intracellularly in the regulation of NAD biosynthesis, and NAMPT inhibitors have been shown to have anti-inflammatory potential in *in vivo* models of inflammation [33,34]. NAD is required for cellular metabolism and is an essential co-factor for enzymes, such as sirtuins (protein deacetylases), and poly (ADP-ribose) polymerases (PARP). These proteins are involved in the transcriptional control of many cellular processes, including the response to cytokines [169,250]. Inhibition of NAMPT-mediated NAD biosynthesis with FK866, down-regulates cytokine production from inflammatory cells *in vitro*, and is reversed by addition of the enzyme product nicotinamide mononucleotide (NMN) [33]. Moreover, accumulation of nicotinamide (NAM) (the substrate of NAMPT) has also been shown to decrease cytokine

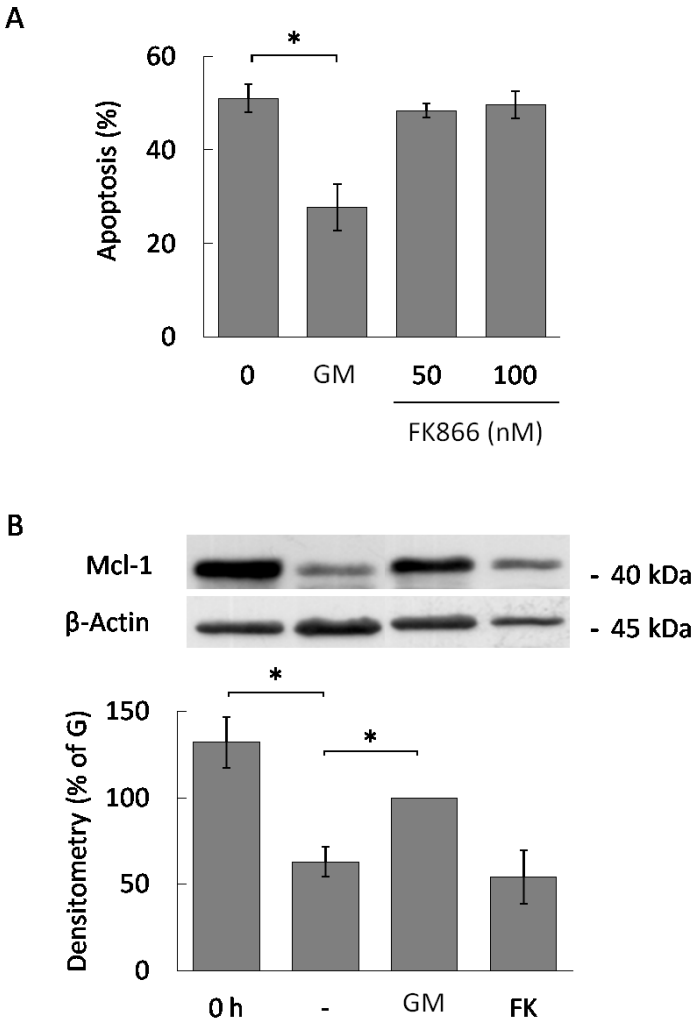

FIGURE 3.11: NAMPT inhibition with FK866 has no effect on neutrophil apoptosis or turnover of Mcl-1. A. Neutrophils were incubated for 17 h with media alone (-), GM-CSF (GM) or 100 nM FK866, before apoptosis was quantified by annexin V binding and PI uptake measured by flow cytometry. Results are representative of 6 experiments. **B**. The effect of NAMPT and its inhibition on turnover of Mcl-1 was assayed by immunoblotting, neutrophils were assayed at 0 h and 5 h with the treatments listed previously. Densitometry represents the mean ±SEM of 6 independent experiments and a representative blot is shown above, *$p<0.05$.

production [35,228]. No studies have yet specifically addressed the effect of NAMPT inhibition on neutrophil function. Here, it is shown that FK866 depletes neutrophil NAD/H and NADP/H stores, which leads to a decrease in the reactive oxidants secreted by these cells in response to receptor-dependent and -independent stimulation. However, this does not cause a concomitant decrease in neutrophil phagocytosis or bacterial killing.

FK866 inhibits the first step in the respiratory burst, reduction of molecular O_2 by the NADPH oxidase complex. O_2^- is then converted into a variety of secondary oxidants, spontaneously, and by the action of other cellular enzymes [1,243]. The NADPH oxidase uses the reducing power of NADPH to reduce oxygen, resulting in $NADP^+$; which is converted back to NADPH via the hexose monophosphate (HMP) shunt. Neutrophil pools of NADP/H are insufficient to fuel the oxidase, and are increased greater than 3-fold upon neutrophil activation with fMLP or PMA [83], requiring new input of phosphorylated NAD. However, FK866 inhibits intracellular production of NAD and NADP, and in this way FK866 may also impede the production of reactive oxidants. These findings are supported by the recent results of Schilling *et al*. [229] who demonstrated a decrease in ROS from monocytes with FK866 treatment, although they found that exogenous NAD did not restore this function, and suggest that FK866 may directly affect NADPH oxidase activity. It has been shown

previously that accumulation of NAM, the substrate of NAMPT, has the potential to suppress production of reactive oxidants [230], so if NAM were to accumulate as a consequence of NAMPT inhibition, it could affect production of oxidants in this manner.

CD16b (FcγRIIIb) is a member of the Fcγ receptor family, which is responsible for the binding and endocytosis of soluble antibody-antigen complexes and IgG coated particles [1,246]. The CD16b isoform is expressed only on neutrophils, where it is present on the cell surface in large numbers of 100,000-200,000 molecules per cell [1,64]. It is attached to the cell membrane via a glycosylphosphatidylinositol (GPI) linkage, which can be easily cleaved by metalloproteinases. The receptor is cleaved during apoptosis, and also during neutrophil activation by cytokines such as GM-CSF [64,251]. However, during activation with such agents, sub-cellular stores of pre-formed receptors can be mobilised, replenishing surface levels [247]. This is confirmed here by flow cytometry, in which GM-CSF treatment resulted in an increase in shedding and a decrease in the intracellular stores of CD16b, whilst overall the surface levels remained unchanged. In this study, FK866 decreased both GM-CSF-induced shedding, and the intracellular stores of CD16b. However, this did not appear to affect surface expression of the formed receptor. Translocation of pre-formed receptors allows for rapid response to stimuli in neutrophils, and in these experiments depletion of the CD16b stores

did not affect the ability of the cells to maintain surface expression of the receptor, but may have led to a down regulation of shedding to conserve the depleted pre-formed receptor stores. In this way, NAMPT inhibition would not be expected to affect neutrophil receptor function, which corresponds with the observation that FK866 does not affect neutrophil phagocytosis of bacteria.

The decrease in reactive oxidants produced by neutrophils, following FK866 treatment, could be beneficial to decrease the tissue damage caused by these cytotoxic products in inflammatory diseases [2]. However, production of ROS is a crucial component of the anti-microbial action of neutrophils, and down-regulation of this system could potentially render patients more susceptible to infections. It was investigated whether inhibition of NAMPT with FK866, had an adverse effect on neutrophil functions important in host defence, and it was shown that FK866 did not decrease neutrophil phagocytosis or killing of *S. aureus*. In view of the importance of ROS in bacterial killing, this lack of inhibition of bacterial killing by FK866, when the inhibitor significantly decreases ROS production, appears difficult to reconcile. However, there is precedent for this in neutrophils of patients with chronic granulomatous disease (CGD), which is a disorder of the neutrophil phagosomal oxidase [97–99]. Patients have impaired ability to generate a respiratory burst, and as such, oxygen dependent microbicidal activity is repressed, rendering neutrophils unable

to kill many microorganisms, leading to recurrent infection. The X-linked form of the disease is the most severe and often patients with this form of the disease have a severe defect of NADPH oxidase activity and severe problems with infections [98]. However, the autosomal recessive form of the disease is more variable and some patients with this form of CGD are only identified by chance or by genetic studies [95,96]. Often, such patients have significantly impaired NADPH oxidase activity, but no problems with infections: i.e. the residual oxidase activity, although decreased compared to healthy controls, is sufficient to sustain intra-phagosomal killing, alongside non-oxidative processes [100,252,253]. In this study, presumably, the residual intra-phagosomal activity of the NAPDH oxidase in NAMPT-inhibited cells was sufficient to create an anti-microbial environment, and alongside O_2 independent killing mechanisms such as release of granule proteases, sufficient bacterial killing was maintained.

In conclusion, these data show that inhibition of NAMPT-catalysed NAD biosynthesis can down-regulate neutrophil activities that have the potential to cause tissue damage in inflammatory disease, without compromising their microbicidal action and thus host defence. These findings, alongside previous work showing that FK866 can decrease production of inflammatory cytokines [33,34], suggest that NAMPT is a potential therapeutic target in inflammatory disease. However, inhibition

of NAD biosynthesis could have wide ranging effects on many cell types, so it will be of interest to monitor the progress of this drug following the recent completion of phase II clinical trials for the treatment of cancer [231,232].

CHAPTER 4: Effects of NAMPT on Neutrophil Functions

4.1 Introduction

Whilst it has previously been reported that NAMPT regulates neutrophil apoptosis [19], the full range of effects of this molecule on other neutrophil functions has not yet been determined. NAMPT expression is upregulated in a variety of acute and chronic inflammatory diseases including RA [18,32,165], and it may contribute to the perpetuation of the inflammatory state in this disease via inhibition of neutrophil apoptosis [19]. There is accumulating evidence that NAMPT acts as an extracellular cytokine-like molecule and growth factor that can stimulate immune cells independently of its enzyme activity [21,22,167]. *In vitro*, exogenous recombinant human NAMPT (rhNAMPT) has been shown to stimulate cells involved in inflammation to express a number of cytokines, such as TNFα, IL-1β, IL-1ra, IL-6 and IL-10 [20].

4.2 Aims

The aims of this chapter were to characterize the *in vitro* responses of control neutrophils to recombinant human (rh)NAMPT, at concentrations observed in inflammatory diseases such as RA. A variety of neutrophil functions were tested to establish whether exogenous NAMPT could, like many other cytokines, prime these cells. This would evaluate whether

NAMPT contributes to the inflammatory potential of neutrophils in chronic inflammatory disease.

4.3 Results

4.3.1 NAMPT does not affect neutrophil surface marker expression

Neutrophil priming, by agents such as GM-CSF, affects the expression of cell surface receptors that facilitate many neutrophil functions, including transmigration and pathogen recognition [50,64,254]. Therefore, expression of some key markers was measured on resting neutrophils, and compared with cells stimulated with either GM-CSF or rhNAMPT (100 ng/mL) for 1 h. The neutrophils were fixed and stained with the appropriate fluorescently-conjugated antibodies before surface receptor expression was assayed by flow cytometry. As observed in the previous chapter, GM-CSF induced significant shedding of L-selectin ($p<0.05$) (Fig. 4.1A) as well as a significant increase in expression of CD11b ($p<0.01$) (Fig. 4.1B). However, NAMPT had no effect on the extracellular expression of these receptors (Fig. 4.1A, B). Neither GM-CSF nor NAMPT caused a significant change in the surface expression of CD16 or CD32 [48] (Fig. 4.1C,D)

4.3.2 NAMPT is not a neutrophil chemoattractant

One of the features of RA is massive leukocyte infiltration into affected joints, via processes that are controlled by the presence of neutrophil

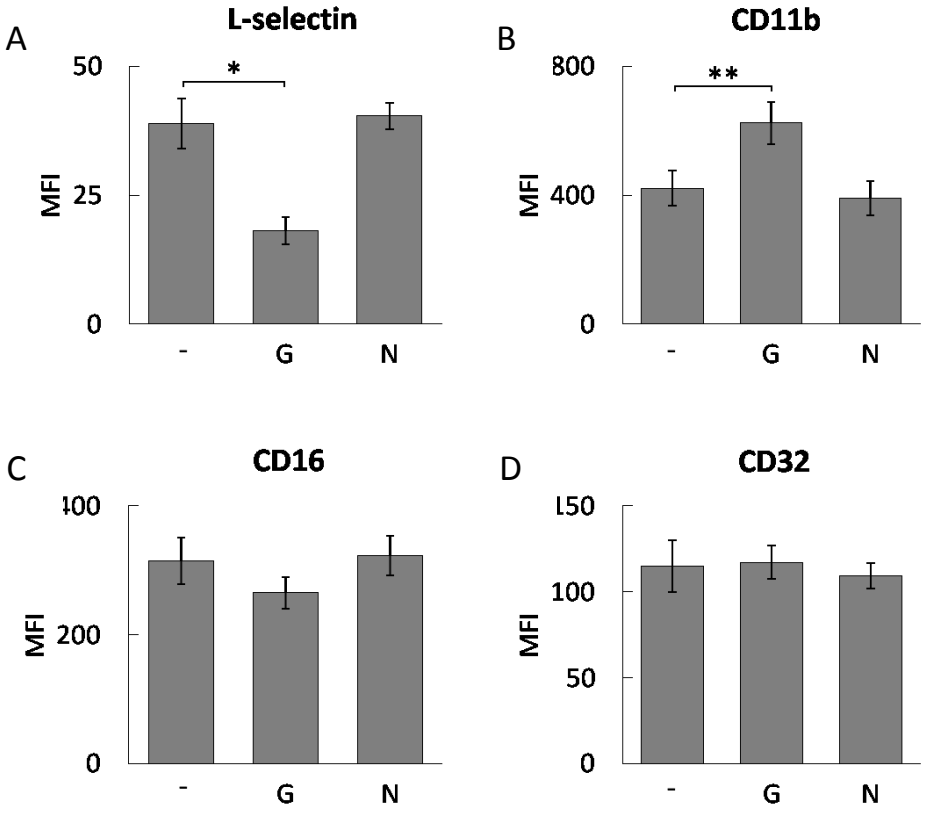

FIGURE 4.1: NAMPT does not affect the surface expression of key neutrophil receptors. Neutrophils were incubated for 1 h with 100 ng/mL NAMPT (N), GM-CSF (G) or media alone (-) and surface expression of L-selectin (**A**), CD11b (**B**), CD16b (**C**) and CD32 (**D**) was measured by flow cytometry. Data represent mean ±SEM of 4 independent experiments, *p<0.05, **p<0.01.

chemoattractants in the joint [7,8,10]. It is well established that NAMPT is elevated in the synovial fluid of RA patients [18,165,218], so it was investigated whether rhNAMPT, at patho-physiological concentrations, was capable of inducing neutrophil chemotaxis. Hanging culture well inserts with a 3 μm polycarbonate filter were suspended in a culture plate containing media and the indicated chemoattractant. Following this, 1 x 10^6 neutrophils were added into the hanging well inserts, and incubated at 37°C for 1 h. The cells that had migrated through the filter towards the chemoattractant were counted using a Multisizer 3 cell counter. 10 nM fMLP was used as a positive control for chemotaxis. Around 50% ($p<0.05$) of the neutrophils transmigrated through the membrane towards fMLP. However, no more cells migrated towards NAMPT at 50 or 100 ng/mL, than were observed to move due to random migration alone (~ 5% migration) (Fig. 4.2).

4.3.3 NAMPT does not prime neutrophils for the respiratory burst

Neutrophils may be primed by exposure to cytokines prior to producing reactive oxidants in response to stimuli [3]. In inflammatory diseases, neutrophils can become inappropriately primed by high levels of circulating cytokines [11]. Priming stimulates the assembly of the NADPH oxidase complex, which catalyses the first step in oxidant production [255]. To examine whether NAMPT, like other cytokines, is capable of priming neutrophils for the respiratory burst, 5 x 10^6 cells were primed with either

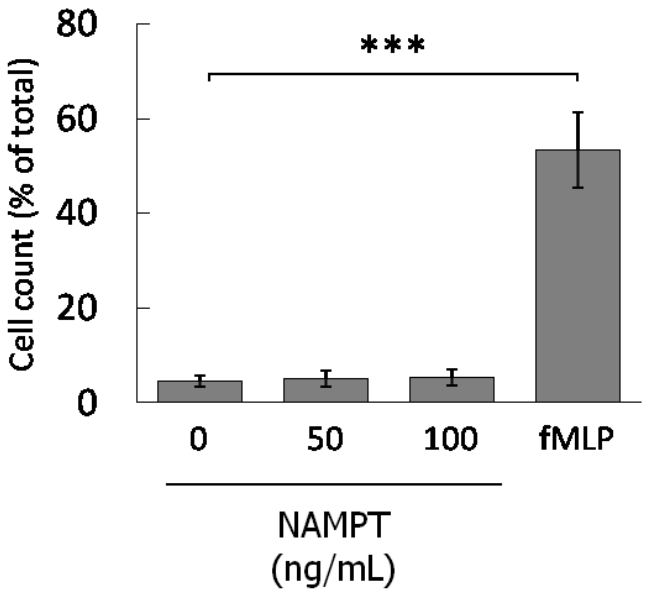

FIGURE 4.2: NAMPT is not a neutrophil chemoattractant. Neutrophils were added to a 3 μM filter chamber suspended above media containing the indicated concentration of NAMPT or fMLP (10 nM) as a positive control for chemotaxis, and incubated for 1 h. The number of migrated Data represent mean ±SEM of 4 independent experiments, ***$p<0.001$.

TNFα (10 ng/mL) for 15 min, or GM-CSF (50 U/mL) for 45 min, alongside the indicated concentrations of rhNAMPT for the same time period. Following this incubation, the respiratory burst was stimulated with fMLP (1 μM) and the production of reactive oxidants was measured by luminol-dependent chemiluminescence.

fMLP stimulated oxidant production from neutrophils increased 7.1-fold ($p<0.01$) with 15 min TNFα priming and 4.5-fold ($p<0.01$) with 45 min GM-CSF priming, but there was no significant increase in the capacity of neutrophils to produce oxidants in response to fMLP following NAMPT treatment for either 15 or 45 min (Fig. 4.3).

4.3.4 NAMPT delays neutrophil apoptosis and stabilizes Mcl-1

Neutrophil apoptosis can be transiently delayed, the major way in which this is controlled in neutrophils is by decreases in the turnover rate of the anti-apoptotic protein Mcl-1 [38]. Pro-inflammatory cytokines, such as GM-CSF, decrease the rate of Mcl-1 turnover by altering the phosphorylation state of the protein [38,108]. Jia *et al*. (2004) [19] have demonstrated that rhNAMPT delays neutrophil apoptosis in a dose-dependent manner *in vitro*. This is corroborated here, as 100 ng/mL rhNAMPT significantly delayed neutrophil apoptosis at 17 h (control 51% ± 4.6%: NAMPT 37 % ± 4.2%, $p<0.05$, $n=6$), measured by flow cytometry of annexin V binding and uptake of PI (Fig 4.4A). As Mcl-1 turnover is the major controlling factor in neutrophil apoptosis, it was then investigated whether

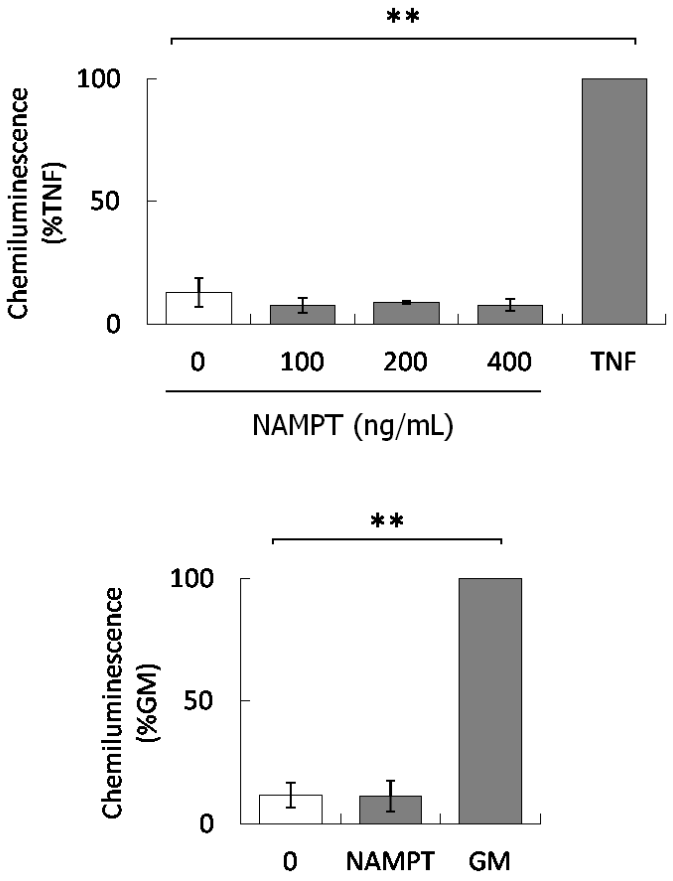

FIGURE 4.3: NAMPT does not prime neutrophils for the respiratory burst. Neutrophils were incubated for 15 min with the indicated concentration of NAMPT or TNFα (10 ng/mL) as a positive control for priming (**A**), or for 45 min with NAMPT (200 ng/mL) alongside GM-CSF (50 U/mL) as a positive control for priming (**B**). Production of ROS was stimulated with fMLP (1 μM) and measured by luminol-enhanced chemiluminescence, displayed as total area under the curve. Data represent mean ±SEM of 3 (**A**) or 4 (**B**) independent experiments, **p<0.01.

rhNAMPT affected the stability of this protein. Figure 4.4B shows that the degradation of Mcl-1 over time (measured at 5 h) is delayed by 100 ng/mL rhNAMPT in a manner comparable to GM-CSF.

4.4 Discussion

NAMPT is elevated in the serum and synovial fluid of RA patients, and has been demonstrated to have a cytokine-like effect on immune cells [18,23,165]. Neutrophils contribute to the inflammatory process in diseases such as RA, and thus it is of interest to indentify factors that can lead to their activation and/or delay their apoptosis [2]. NAMPT has been shown previously to delay constitutive neutrophil apoptosis [19], so it was investigated whether it exerts any other pro-inflammatory effects on these cells. NAMPT, at the range of serum concentrations observed in inflammatory disease, was found to have little effect on neutrophil functions, but it did delay neutrophil apoptosis by delaying the turnover of Mcl-1.

NAMPT did not stimulate any changes in the surface expression of the key cell surface markers tested, suggesting that these cells are not primed by NAMPT. Further evidence that NAMPT is unable to prime neutrophils came from the inability of NAMPT treated cells to mount a respiratory burst. Neutrophils pre-treated with rhNAMPT alone produced no more ROS in response to fMLP than unprimed cells, in comparison to a large upregulation observed with GM-CSF or TNFα priming.

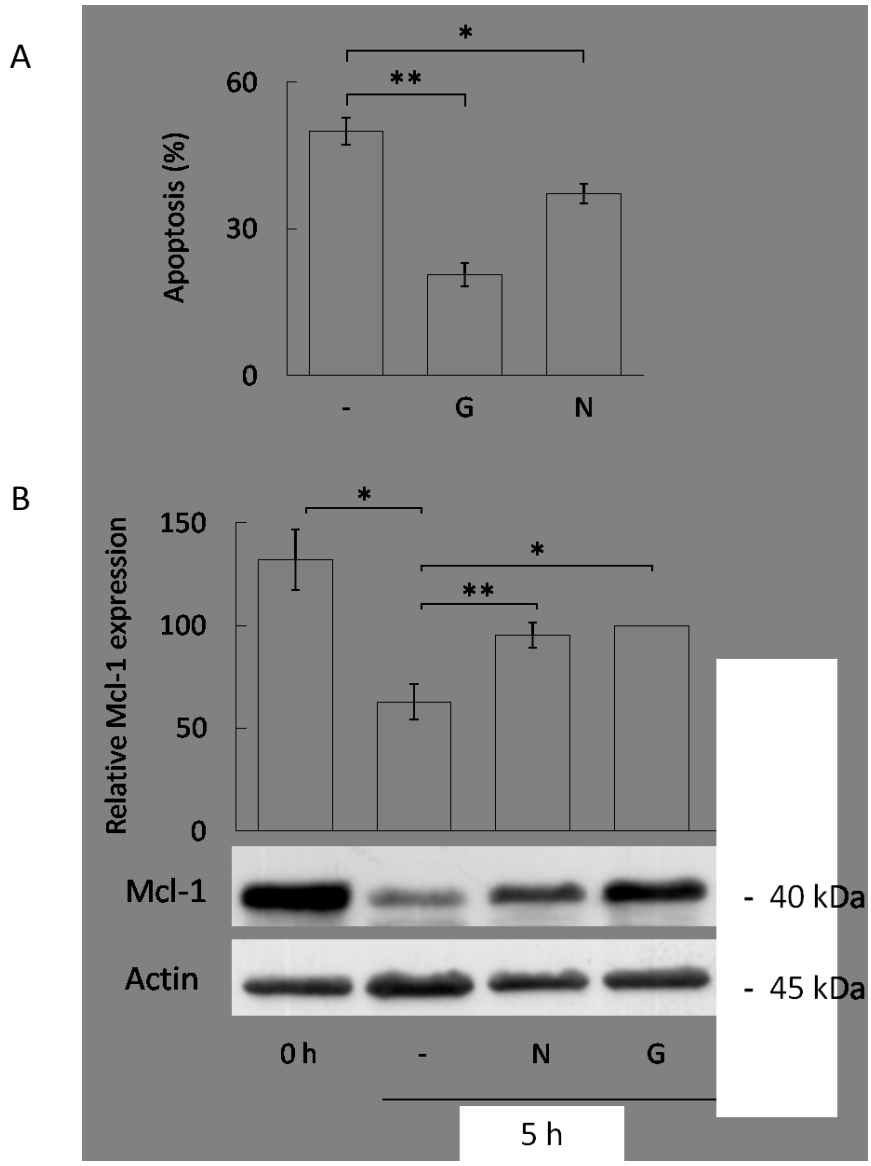

FIGURE 4.4: NAMPT delays neutrophil apoptosis and the turnover of anti-apoptotic Mcl-1. A. Neutrophils were incubated with 100 ng/mL NAMPT (N) or GM-CSF (G) as an anti-apoptotic control for 17 h, then apoptosis was quantified by flow cytometry as binding of annexin V and uptake of PI. Data represent mean ±SEM of 4 independent experiments. **B.** Turnover of Mcl-1 was quantified by immunoblotting; neutrophils were incubated in either media alone (-), or with 100 ng/mL NAMPT (N) or GM-CSF (G) for 5 h. Relative Mcl-1 expression was quantified by comparison to Actin. Data represent mean ±SEM of 6 independent experiments, *$p<0.05$, **$p<0.01$.

Neutrophil priming facilitates assembly of the multi-component NADPH oxidase complex at the plasma membrane, in readiness to mount a respiratory burst when a stimulus is encountered. Malam *et al*., (2011) [224], suggested that rhNAMPT does prime (but not activate) neutrophils for the respiratory burst, by promoting translocation of two of these enzyme subunits, p40 and p47, to the cell membrane via phosphorylation of p40. In their study, the generation of ROS was assayed using isoluminol, which measures extracellular oxidant production only, whereas here luminol was used to measure total intra- and extracellular oxidant production. The extracellular oxidant portion enhanced with isoluminol is reported to constitute less than 22% of the total cellular oxidants, and there is precedent for the two parts of the chemiluminescence signal being differentially modified by the same treatment [256]. This may go some way to explain why Malam *et al*. [224] observed a priming effect with rhNAMPT that was unable to be reproduced here.

It was also concluded that NAMPT does not act as a neutrophil chemoattractant at patho-physiologically relevant concentrations. This is in disagreement with the work of Hong *et al*. (2008) [257], as they found rhNAMPT to be a rat neutrophil chemotactic factor *in vitro*. Hong *et al*. labeled rat peritoneal neutrophils with calcein AM, and assayed transmigration towards rhNAMPT (10 ng/μL) through a bovine albumin

coated filter. The number of transmigrated cells was assayed via fluorescence emission at 517 nm [257]. They suggested that rhNAMPT at 10 ng/μL acts as a rat neutrophil chemotactic agent, however, this concentration is 100 times that observed during inflammation *in vivo* [18,165,218]. The discrepancy between these findings and the experiments in this report may be accounted for by the high concentration of rhNAMPT used and by the cross-species experimentation.

In contrast to the lack of effect on other neutrophil functions tested, NAMPT significantly delayed neutrophil apoptosis by delaying turnover of the anti-apoptotic protein Mcl-1. Pro-inflammatory cytokines such as GM-CSF decrease the rate of Mcl-1 turnover by altering the phosphorylation state of the protein, transiently delaying neutrophil apoptosis [38,108]. Control of this process is crucial for effective resolution of inflammation, and deregulated neutrophil apoptosis has been implicated in numerous chronic inflammatory conditions such as RA [2,133]. Jia *et al*. (2004) [19] have also demonstrated that rhNAMPT delays neutrophil apoptosis in a dose dependent manner *in vitro*. They suggest this is due to decreased activity of caspases-8 and -3. This cascade of caspase activity is initiated by death receptor engagement, and brings about apoptosis of the cell, which includes cleavage of Mcl-1 by caspase-3. Thus the decrease in caspase activation in response to NAMPT observed by Jia *et al*. corresponds with the observation here that NAMPT

delays the turnover of Mcl-1 in neutrophils. NAMPT may contribute to the dysregulation of neutrophil apoptosis observed in chronic inflammation, which exacerbates the damage caused in inflammatory diseases such as RA. Thus, although here NAMPT appeared to have little stimulatory effect on pro-inflammatory neutrophil functions, it may contribute to the perpetuation of inflammation by delaying neutrophil apoptosis, when present at high concentrations such as those observed in the rheumatoid joint [18].

CHAPTER 5: Dynamics of NAMPT Expression by Neutrophils

5.1 Introduction

Until recently, neutrophils were considered to be terminally differentiated cells with little capacity for active gene expression. However, it is now understood that activated neutrophils express a variety of cytokines and chemokines [17,258,259]. NAMPT has been shown to be upregulated in neutrophils in response to IL-1β, TNFα and LPS [19], and it is also upregulated in a number of other immune cells in response to endotoxin and a variety of inflammatory cytokines [23]. The NAMPT gene contains a binding site for the transcription factor NF-κB [176], which is activated by pro-inflammatory cytokines, such as TNFα and IFNγ [260]. The NF-κB binding site is located at the distal NAMPT promoter alongside NF-1 binding sites, whereas AP-2 binding sites are located at the proximal promoter, with AP-1 binding sites found throughout both regions [176].

NAMPT protein is detectable in the circulation and correlates with leukocyte counts [184], and it is elevated in the serum and synovial fluid of patients with rheumatoid arthritis [18,32,165]. It is suggested that NAMPT can be secreted from cells, and this has been termed extracellular or eNAMPT. The mechanism by which secretion occurs is not well understood, and as NAMPT lacks an identifiable signal peptide sequence that is commonly seen in hematopoietic cytokines and necessary for extracellular secretion [167], it has been suggested that eNAMPT may be

released during cell death and lysis [171,212]. However, it has been shown that NAMPT is secreted by the 3T3-L1 adipocyte cell line during culture, via a non-classical pathway that is independent of the endoplasmic reticulum (ER)-Golgi system or microvesicles [211].

5.2 Aims

NAMPT is expressed by a number of immune cells, including neutrophils, can be dynamically regulated in response to cytokines and is found in the circulation. It has been suggested that NAMPT acts as an extracellular signalling molecule, but the mechanism of NAMPT secretion is yet to be elucidated [23]. The aims of this chapter were to characterise expression of NAMPT RNA and protein by neutrophils, and to investigate whether NAMPT protein is secreted from neutrophils *in vitro*. The effects of cytokine stimulation on NAMPT expression by neutrophils were also investigated.

5.3 Results

5.3.1 Effects of cytokine stimulation on NAMPT RNA expression from neutrophils

It has been shown previously that neutrophils express NAMPT mRNA transcripts in response to the inflammatory stimuli LPS, TNFα and IL-1β, and that NAMPT protein expression is similarly induced by stimulation with LPS [19]. Here, it was investigated whether other pro-inflammatory

cytokines are capable of inducing transcription of NAMPT in healthy neutrophils *in vitro*. Neutrophils were incubated with the indicated stimuli for 1 h at 37°C with gentle agitation. Following this the cells were lysed with Trizol for isolation of RNA (as described in section 2.2.9). NAMPT RNA transcripts were quantified following conversion to cDNA and subsequent qPCR (as described in 2.2.10). Figure 5.1 shows a modest, but significant increase in NAMPT RNA transcripts in response to all of the cytokines tested, namely GM-CSF, IL-1β, IL-6, IL-8 and TNFα, and also with bacterial LPS. rhNAMPT did not induce expression of NAMPT mRNA by neutrophils.

5.3.2 Effects of cytokine stimulation on NAMPT protein expression from neutrophils

The dynamics of NAMPT protein expression, quantified by western blot, showed more variability between donors than NAMPT mRNA expression when cells were treated with the same inflammatory stimuli. Neutrophils were incubated with the indicated stimuli at 37°C with gentle agitation, and then lysed in boiling Laemmli buffer at the indicated time-points. Samples were then subjected to SDS-PAGE and immunoblotting for NAMPT. NAMPT expression generally appeared to increase over time in neutrophils (Fig. 5.1B), although this did vary between donors. NAMPT protein expression in response to stimuli was also highly variable, and no

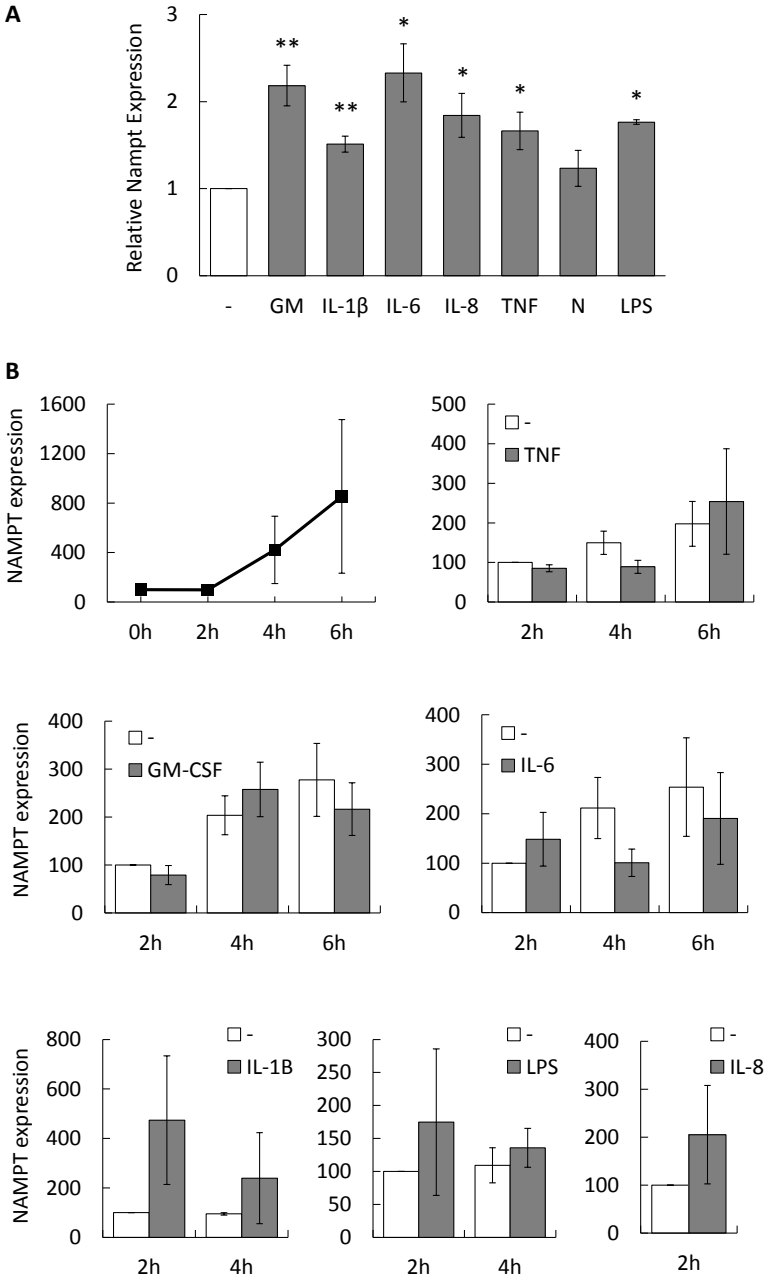

FIGURE 5.1: NAMPT mRNA transcript is increased by exposure to cytokines, but protein levels are variable and not significantly altered. **A.** Neutrophils were incubated for 1 h with the indicated cytokine or NAMPT (N) alongside media alone (-) and NAMPT expression was assayed by qPCR. Data represent mean ±SEM of 6 independent experiments. **B.** Neutrophils were incubated with the indicated cytokine for the indicated time alongside media alone (-), then NAMPT protein expression was analysed by immunblotting. Results shown as percentage expression compared to control. Data represent mean ±SEM of at least 3 independent experiments, *$p<0.05$, **$p<0.01$.

cytokine tested induced a significant upregulation in NAMPT protein, although IL-1β appeared to increase NAMPT expression at 2 h (Fig. 5.1B).

5.3.3 NAMPT expression is under the control of transcription factor NF-κB in neutrophils

The NAMPT gene encodes a binding site for the transcription factor NF-κB alongside others [176]. Therefore, the selective NF-κB inhibitor Bay11 was used to investigate the dynamics of NAMPT expression from neutrophils by this transcription factor. Bay11 inhibits NF-κB activity by inhibiting phoshorylation of IκBα induced by TNFα, but it does not affect constitutive IκBα phosphorylation [261]. Inhibition of NF-κB activity with this agent induced a down-regulation of constitutive NAMPT protein expression in neutrophils with little protein detectable by 6 h (Fig. 5.2). This suggests neutrophils do constitutively express NAMPT *de novo* over time and that despite the presence of numerous transcription factor binding sites in the NAMPT promoter, NAMPT gene expression is primarily induced via NF-κB.

5.3.4 NAMPT secretion from neutrophils

NAMPT is expressed by neutrophils and it is suggested that NAMPT can be secreted; however the mechanism by which this occurs is not well understood. Here, neutrophil culture supernatants, alongside cell pellets, were immunoblotted for NAMPT in the presence of the priming agent GM-CSF and cytochalasin B (CB). CB disrupts the cellular actin

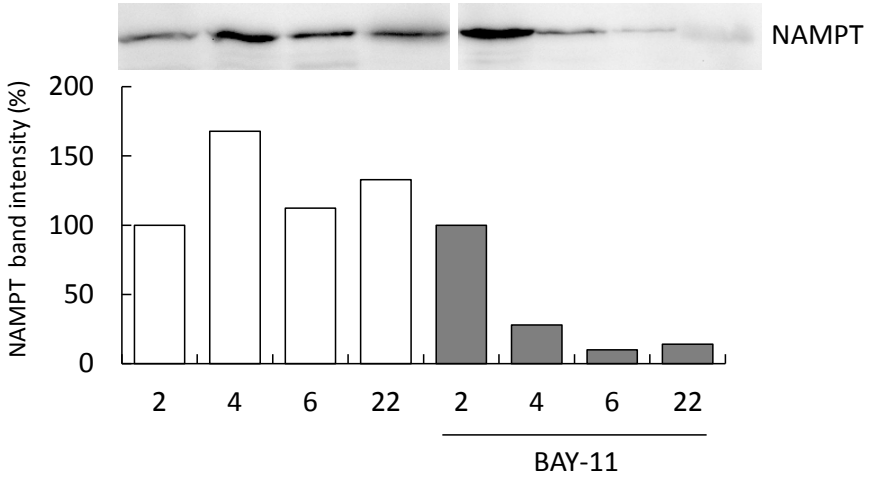

FIGURE 5.2: NF-κB inhibition decreases constitutive expression of NAMPT from neutrophils. Neutrophils were incubated for the indicated time (h) (x axis) in the absence and presence of the NF-κB inhibitor BAY-11, before cell lysates were subjected to immunoblotting for NAMPT. Representative blot and densitometry shown.

cytoskeleton and facilitates degranulation, one of the major mechanisms by which neutrophils secrete proteins. NAMPT was not detected in the supernatant from these samples; however, GM-CSF and CB in combination decreased the amount of intracellular NAMPT. This decrease may be due to degradation of the protein upon the co-release of NAMPT and intracellular proteases, as indicated by the increase in immune-reactive lower molecular weight products. The supernatant was also probed for the granule protein myeloperoxidase (MPO) as a positive control (Fig. 5.3A). To detect whether NAMPT was released from neutrophils at levels below the detection limit of the western blot assay, protein was precipitated from the neutrophil culture supernatants using ice cold acetone and then re-suspended in Laemmli buffer. This was done for samples incubated over the indicated time period with GM-CSF and TNFα priming. Figure 5.3B shows that 52 kDa NAMPT was detected albeit faintly, but a more intense signal was detected at 30 kDa. This may be a degradation product, as lower molecular weight, immune-reactive bands were also present in proteins isolated from cell lysates, and were decreased in intensity by addition of the serine protease inhibitor AEBSF. This 30 kDa species was only detected in culture supernatant and not observed in neutrophil whole cell lysates, although it was also present in low levels the 0 h samples.

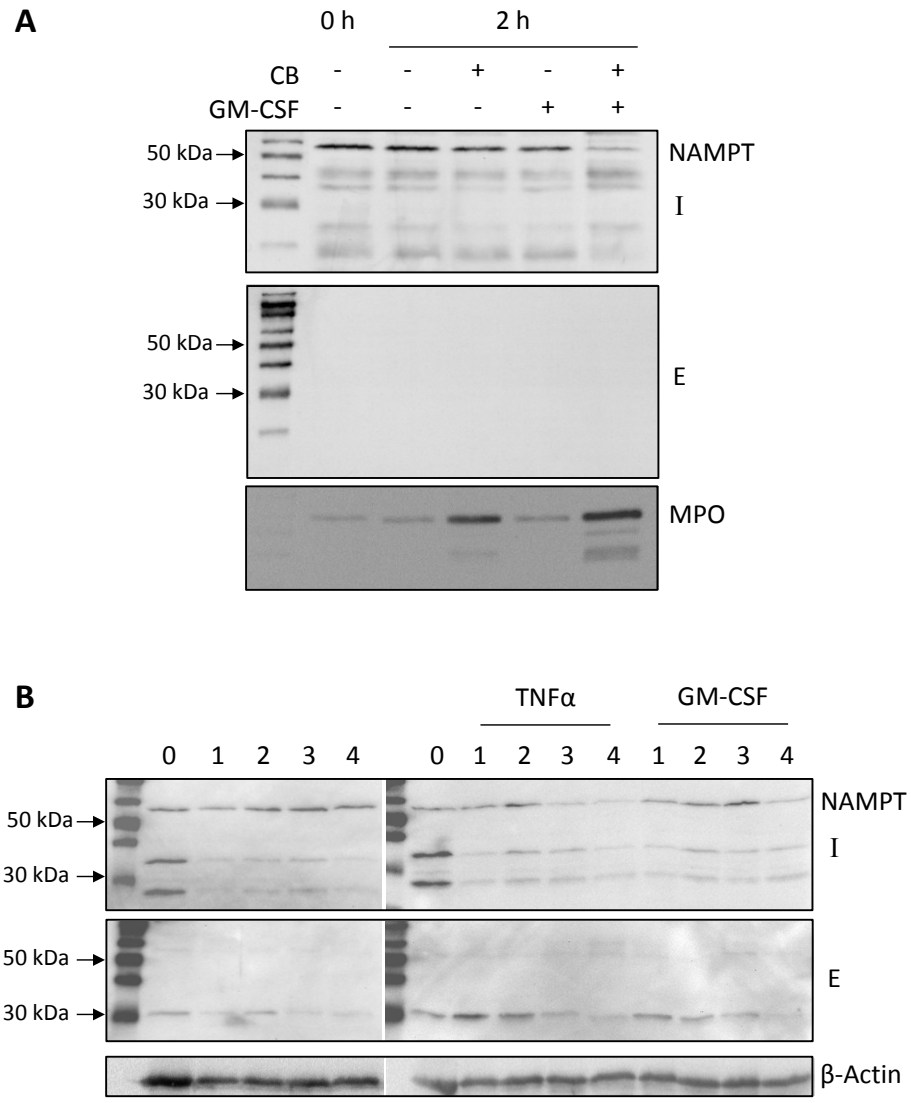

FIGURE 5.3: Whole NAMPT is only detected in concentrated culture supernatants at very low levels. A. Neutrophils were incubated for 2h in the presence and absence of both cytochalasin B (CB) and GM-CSF, the cell lysates and the culture supernatant were then subjected to immunoblotting for NAMPT, immunoblotting for MPO was also carried out as a positive control for degranulation. **B**. Neutrophils were incubated for the indicated time (shown above blot) in the presence and absence of either TNFα or GM-CSF. The protein content of the culture supernatant was precipitated with acetone, and re-dissolved in 50 µL Laemmli buffer; this was subjected to immunoblotting for NAMPT alongside the cellular protein contents. I – intracellular, E – extracellular. Representative blots shown.

5.3.5 NAMPT expression in neutrophils from patients with rheumatoid arthritis

NAMPT has been reported to be elevated in the serum and synovial fluid of RA patients, and serum NAMPT correlates with disease activity [18,32,165]. Here it was investigated whether expression of NAMPT from neutrophils correlated with changes in disease activity (measured by DAS) during treatment and also whether NAMPT expression correlated with the expression of the key inflammatory cytokine TNFα, known to be expressed by inflammatory neutrophils. RNA was isolated from the peripheral blood neutrophils of 5 RA patients (RA1-5) referred for anti-TNF therapy, prior to the commencement of treatment and for 3 of these patients (RA1-3), again at a time point at least 6 months following commencement of treatment. Table 5.1 gives the disease activity data for each patient before and after treatment along with the European League against Rheumatism (EULAR) response classification. RNA was also isolated from the peripheral blood neutrophils of 2 patients with newly diagnosed RA, samples were collected at first appointment following diagnosis, before the commencement of DMARD therapy (Table 5.2).

Expression of neither NAMPT nor TNFα correlated with the patient disease activity, measured by DAS. However pre-treatment, the expression of NAMPT does significantly correlate with the expression of TNFα from neutrophils ($p<0.05$ Pearson $r^2=0.78$). Although post-

treatment, this correlation does not hold (Fig.5.4A). The expression of both NAMPT and TNFα from neutrophils increased slightly post-treatment in this small sample set, although this was not significant and the effect did vary between patients (Fig. 5.4B). A comparison between the expression of these molecules from the blood neutrophils of early arthritis patients and those with more established disease was carried out; there was no difference in the average NAMPT expression between these groups, and very little difference in the expression of TNFα (Fig. 5.4C).

Patient	DAS		EULAR response	Notes
	Pre-treatment	Post-treatment		
RA1*	6.86	5.99	None	Etanercept non-responder. Switched to Rituximab
RA2*	6.95	1.55	Good	Maintained on Adalimumab
RA3*	6.69	5.23	Moderate	Injection site reaction to Etanercept, switched to Adalimumab but did not respond well. Now on Rituximab
RA4	4.02	3.57	n/a	Maintains good response to Sulfasalazine
RA5	6.19	2.64	Good	Maintained on Etanercept

TABLE 5.1: RA patients qualifying for anti-TNF treatment from whom samples were analysed (*sample pre- and post-treatment)

Patient	Treatment		Current DAS	Notes
	At sample	Current		
eRA1	NSAID	Methotrexate	4.03	In remission
eRA2	NSAID	Methotrexate	3.21	In remission

TABLE 5.2: Early RA patients, samples collected following diagnosis

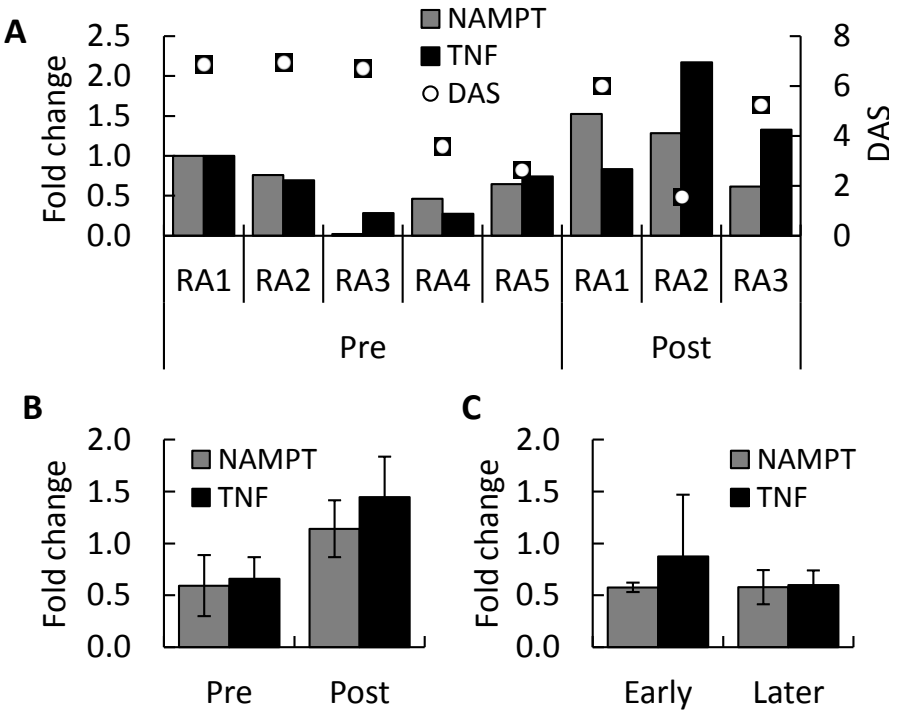

FIGURE 5.4: Comparison of NAMPT and TNFα expression from the blood neutrophils of RA patients at different stages of disease. RNA was isolated from peripheral blood neutrophils and analysed for expression of NAMPT and TNFα by qPCR. **A.** Samples were collected from RA patients (n=5 RA1-5) referred for anti-TNF therapy prior to commencement of treatment, and then at a time point >6 months post start of treatment for 3 patients (RA1-3). Expression of NAMPT correlated with the expression of TNFα pre-treatment ($p<0.05$ Pearson $r^2=0.78$), but not at the post-treatment timepoint. The expression of NAMPT and TNFα did not correlate with the DAS of the patients. **B.** The average expression of NAMPT and TNFα was increased slightly post treatment (n=3 not significant). Data represent mean ±SEM of at least 3 independent samples **C.** Blood neutrophils were collected from newly diagnosed RA patients (early), and NAMPT and TNFα expression was compared to that of patients with more established disease (later) (from **A.** and **B.**), there were no significant changes in expression. Data represent mean ±SD of 2 independent samples.

The concentration of NAMPT is reported to be higher in the synovial fluid than in the serum of RA patients [18]. To investigate whether this increase in NAMPT concentration in synovial fluid is mirrored by increases in cellular content in synovial fluid neutrophils, neutrophils from paired synovial fluid and peripheral blood samples were lysed and the contents subjected to immunoblotting for NAMPT. Figure 5.5 shows that NAMPT expression in neutrophils isolated from synovial fluid was significantly higher than in cells isolated from the peripheral blood of the same patients (30-fold increase, $n=8$ $p<0.01$ by Wilcoxon signed-rank test). However, the magnitude of this increase did vary between patients.

5.4 Discussion

NAMPT expression by immune cells in response to cytokines is well documented [19,32,176,185,186,213,262], and it has been shown that NAMPT expression can be induced in neutrophils by IL-1β, TNFα and LPS [19]; these authors suggest that induction of expression by LPS occurs via initial expression of IL-1β, and subsequent stimulation of NAMPT. As with the data presented here, these increases in NAMPT expression appear to be more apparent at the mRNA level than they do at the protein level. Here, I have found that the degradation of the NAMPT protein and the large variation in expression between individuals makes it difficult to draw conclusions about NAMPT protein expression in neutrophils. Cytokine treatment does induce significant upregulation of NAMPT

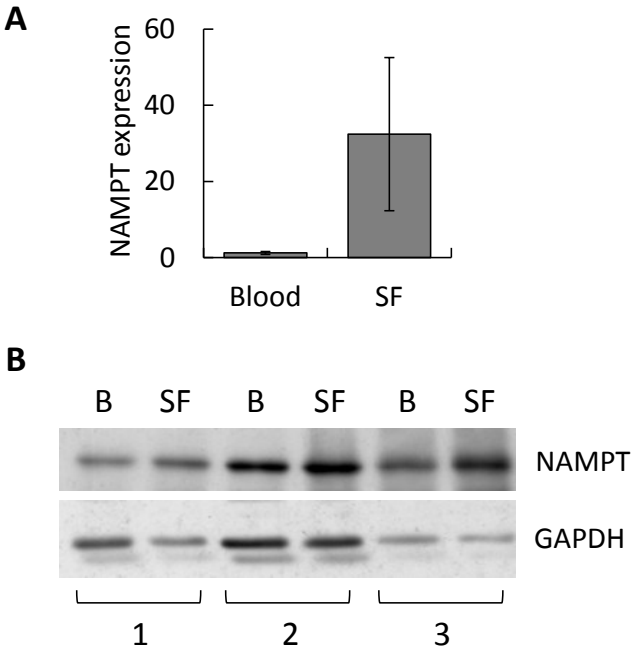

FIGURE 5.5: NAMPT expression is increased in neutrophils isolated from synovial fluid of RA patients as compared to paired peripheral blood neutrophils. Protein was isolated from the peripheral blood (B) and synovial fluid (SF) of RA patients and subjected to immunoblotting for NAMPT. NAMPT expression was significantly elevated in the synovial fluid neutrophils (**A.**) $p<0.01$ by Wilcoxon signed-rank test. Data represent mean ±SEM of 8 independent samples and a representative blot is shown in **B.** (1, 2 and 3 represent individual patients).

transcripts, but only to a maximum of 2-fold, and it does not appear to be mirrored by changes in protein expression of NAMPT. Although it does not appear to be dynamically regulated by exposure to cytokines, NAMPT is constitutively expressed by healthy donor neutrophils, and as such it is likely that it is important for neutrophil function. NAMPT expression increased over time in culture, and could be blocked by inhibition of the transcription factor NF-κB, which binds to the distal promoter of the NAMPT gene. Although the NAMPT gene contains binding sites for a number of other transcription factors, namely NF-1 and AP-1 and -2, it appears that blocking activation of NF-κB alone is sufficient to almost completely ablate NAMPT protein expression by 6 h. This indicates that this is the major transcription factor for constitutive NAMPT expression in neutrophils.

There is conflicting data published about the secretion of NAMPT from various cell types, and it is suggested that because NAMPT lacks a signal sequence for secretion [167], extracellular NAMPT is only released upon cell death and lysis [171,212]. However, one study showed that NAMPT is actively secreted from a 3T3-L1 adipocyte cell line, and this was not blocked by addition of (ER)-Golgi-dependent secretion inhibitors, nor was it thought to be secreted via microvesicles: thus, it was concluded that secretion occurs via a non-classical pathway, using a number of distinct secretory mechanisms [211]. It has been shown that NAMPT has

extracellular nicotinamide phosphoribosyltransferase activity [26,27], and that it can act as an extracellular cytokine in the absence of its enzymatic function. It has been proposed that monomers and enzymatically inactive forms of NAMPT are capable of stimulating expression of IL-6 and promoting survival of macrophages [22]. Monomeric NAMPT has a relative molecular mass of 52 kDa, and so it is unlikely that the 30 kDa product detected in the neutrophil supernatant is capable of dimerisation or has any enzyme activity. However, if monomeric NAMPT is capable of signalling in the absence of enzyme activity, then this isoform of NAMPT may also have signalling function. However, given the abundance of proteases within neutrophils, it is likely that the lower molecular weight mass detected by immunoblotting are non-specific degradation products.

Given the high level of NAMPT expression by other cell types [19,32,176,185,186,213,262], and the lack of extracellular NAMPT detected in neutrophil culture supernatants, it may be concluded that neutrophils are not major contributors to the high concentration of NAMPT observed in the serum and synovial fluid of RA patients [18,32,165]. However it has been observed that serum NAMPT correlated with leukocyte counts in children, suggesting that *in vivo* autocrine or paracrine signalling may stimulate secretion from these cell types [184]. NAMPT is constitutively expressed in healthy donor neutrophils, and adding cytokines to

neutrophil cultures in isolation had little effect on this expression here. However, in this preliminary study of RA patient neutrophils, the mRNA expression of NAMPT did correlate with that of TNFα from the peripheral blood neutrophils of RA patients whose disease was currently not well controlled (those referred for anti-TNF therapy). TNFα is an inflammatory cytokine expressed by neutrophils, known to play a crucial role in the progression of inflammatory arthritis, and the drugs that target this molecule are very successful at treating RA in the majority of patients [146,147,150]. This suggests that NAMPT may play a similarly pivotal role in these processes. Although, conversely the expression of neither NAMPT nor TNFα from neutrophils correlated with disease activity (DAS) in these patients, and the expression of both molecules was slightly increased following commencement of a successful treatment regimen. RA is a heterogeneous disease, driven by a variety of inflammatory cytokines, and the mechanisms of chronic inflammation and its abatement by various disease modifying drugs (DMARDs) is incompletely understood. Often even those patients whose disease is successfully modified by anti-TNF therapy do not show a decrease in their serum TNFα concentration [263], and it has been suggested that membrane bound TNFα on the surface of cells is more important to the progression of RA than that released into the circulation [264].

In this study NAMPT was however significantly elevated in the synovial fluid neutrophils of RA patients compared to paired peripheral blood cells. Suggesting that in the multi-cellular inflammatory environment the combination of stimuli can lead to upregulation of NAMPT in activated neutrophils. Synovial fluid neutrophils have been previously primed by exposure to cytokines and undergone transmigration towards the inflammatory site, and it has been shown that transmigration itself can affect the neutrophil expression profile (Lam *et al*., unpublished observation). This small scale investigation of NAMPT expression in RA patient neutrophils, suggests that NAMPT is regulated in a similar manner to TNFα, and that NAMPT expression may be increased during neutrophil activation. However this preliminary study would require expansion before any firm conclusions could be drawn about the contribution of NAMPT to the function of neutrophils during inflammatory disease.

CHAPTER 6: Effects of NAMPT and NAMPT inhibition on the Neutrophil Expression Profile

6.1 Introduction

It is proposed that NAMPT can be considered as a cytokine because it is released by immune cells and can stimulate other immune cells. It has been shown that dimeric, extracellular (e)NAMPT maintains its nicotinamide phosphoribosyltransferase activity [26,196], but there is also convincing evidence for NAMPT acting as a monomeric extracellular, cytokine-like molecule in the absence of this enzymatic activity [21,22]. However, it is unclear whether the proposed cytokine-like activities of NAMPT are independent of its role in NAD biosynthesis, and no NAMPT receptor has yet been identified [18,19]. It is now appreciated that neutrophils undergo active gene expression in response to stimulation and up-regulated gene products include regulators of inflammation together with cytokines and chemokines that can all enhance and regulate the immune response [258,259]. Circulating NAMPT is elevated during inflammation, but little has been published about the effects of NAMPT on neutrophil gene expression. It is unknown (a) whether NAMPT, like many other cytokines, can activate neutrophil intracellular signalling pathways leading to activated gene expression, or (b) if its role in NAD biosynthesis is important for these processes. The functions of a number of key enzymes involved in chromatin remodelling and control of

transcription (such as sirtuins and PARPs) are NAD-dependent, and I have demonstrated previously that inhibition of NAMPT depletes neutrophil intracellular NAD (Chapter 3). Thus, it may be hypothesised that inhibition of NAMPT and the subsequent decrease in intracellular NAD availability, would affect gene expression in neutrophils.

6.2 Aims

The aims of this chapter were to characterise the effects of NAMPT inhibition on cytokine-induced intracellular signalling leading to gene expression, in order to better understand the requirement of NAD in the activation of transcription in these cells. A secondary aim of this chapter was to investigate whether exogenous rhNAMPT is capable of activating intracellular signalling and transcription in neutrophils, even though a surface receptor for this molecule has not yet been identified.

6.3 Results

6.3.1 Effects of NAMPT and NAMPT inhibition on TNFα expression in neutrophils

Expression of the key inflammatory cytokine TNFα by neutrophils is induced by a number of pro-inflammatory factors, including bacterial LPS and TNFα itself. Elevated expression of TNFα is observed in inflammatory diseases such as RA [265,266] and this observation is the basis for the therapeutic use of TNF inhibitors. It has been reported that, in a

mouse model of inflammation, NAMPT inhibition decreased circulating TNFα levels and the severity of arthritis, and also down-regulated the secretion of TNFα (plus IL-1β and IL-6) from human monocytes [33]. To identify whether FK866 also affected TNFα expression in neutrophils, mRNA was isolated from healthy control neutrophils treated for 1 h with FK866 (100 nM) or media alone, and cDNA generated was subjected to quantitative PCR (qPCR) to measure expression of TNFα. Neutrophils treated with FK866 expressed significantly less TNFα mRNA in response to 1 h treatment with either TNFα itself (n=5, $p<0.001$) or LPS (n=3, $p<0.01$). TNF-induced expression of TNFα was decreased to near basal levels when NAMPT was inhibited (Fig. 6.1A). However, adding exogenous rhNAMPT to neutrophils for 2 h had no effect on expression of TNFα mRNA, or the mRNA expression of other cytokines tested (IL-1β, IL-8 and ICAM1) (Fig. 6.1B).

6.3.2 Effects of NAMPT and NAMPT inhibition on activation of NF-κB

TNFα expression is under the control of the NF-κB signalling pathway, and NAMPT has been shown to activate the transcription factor NF-κB in other cells such as macrophages and endothelial cells [24]. It was investigated here whether NAMPT could stimulate this pathway in neutrophils, and whether inhibition of NAMPT affected TNFα expression via this pathway in neutrophils. Activation of NF-κB was determined by immunoblotting

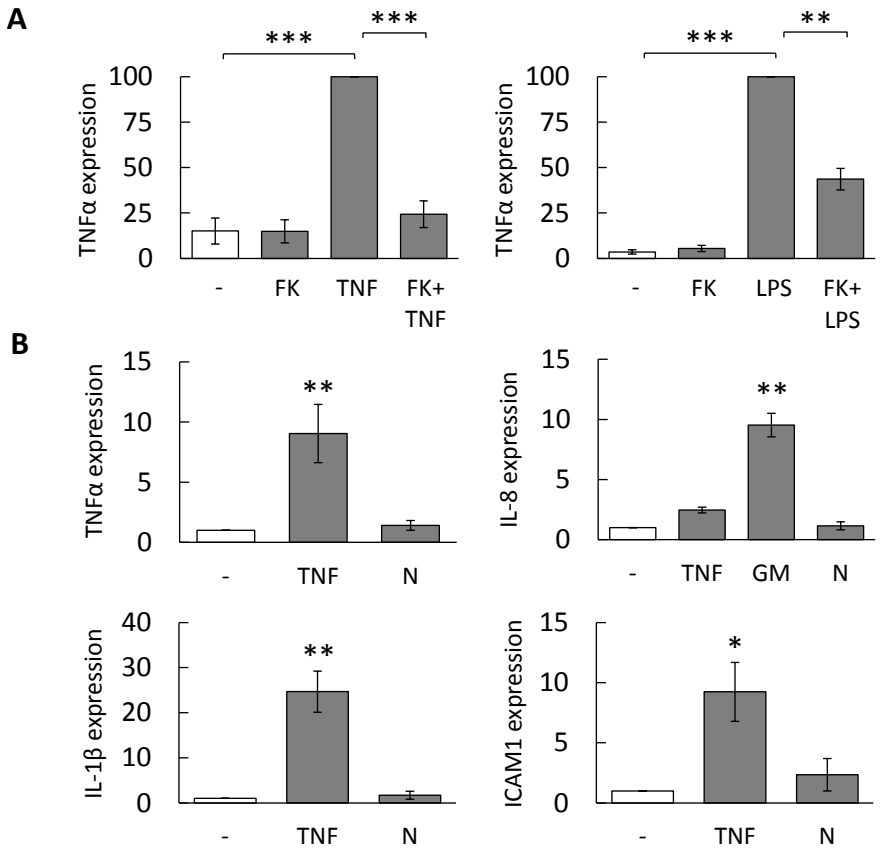

FIGURE 6.1: Effects of NAMPT and NAMPT inhibition on expression of TNFα and other cytokines in neutrophils. A. qPCR measured expression of TNFα from neutrophils either untreated (-) or stimulated by TNFα (n=5) or bacterial LPS (n=3) over 1 h is significantly decreased by a 1 h pre-incubation with 100 nM FK866 (FK). Data expressed as a percentage of TNFα or LPS stimulated expression and represents mean ±SEM of multiple independent samples as indicated **B.** 1 h TNFα but not rhNAMPT (N) (100 ng/mL) significantly induced expression of TNFα, IL-1β and ICAM1 (n=3). 1 h GM-CSF (GM) significantly induced expression of IL-8, and rhNAMPT did not (n=4). Data expressed as fold change in expression from untreated (-) and represents mean ±SEM of multiple independent samples as indicated *p<0.05, **p<0.01, ***p<0.001.

for the phosphorylated form of the p65 subunit of the transcription factor. In initial experiments, neutrophils were stimulated for 5 min or 45 min with rhNAMPT (100 ng/mL) or TNFα (10 ng/mL) as a positive control. At 5 min TNFα significantly induced a 15-fold increase in phosphorylation of p65; there was a trend towards increased phosphorylation by NAMPT, although this was variable (Fig 6.2A left panel). Following 45 min of culture greater constitutive phosphorylation of p65 was observed and the response to TNFα was also more varied; the small stimulatory effect of NAMPT seen at 5 min did not occur at 45 min (Fig 6.2A right panel). It has been demonstrated previously that the product of the NAMPT enzyme reaction, NMN, can also stimulate NF-κB phosphorylation in macrophages [24], suggesting that NAMPT enzymatic activity is important for NF-κB activation in this cell. Due to the effects of NAMPT inhibition on expression of TNFα shown in Figure 6.1A, it was investigated whether inhibition of NAMPT with FK866 could affect the phosphorylation of p65 in neutrophils. Pre-incubating neutrophils for 30 min with FK866 (100 nM), led to a marked decrease in the phosphorylation of p65 in response to a 5 min stimulation with TNFα (10 ng/mL) (Fig. 6.2B). This supports the observation that NAMPT enzyme activity is required for activation of TNFα expression.

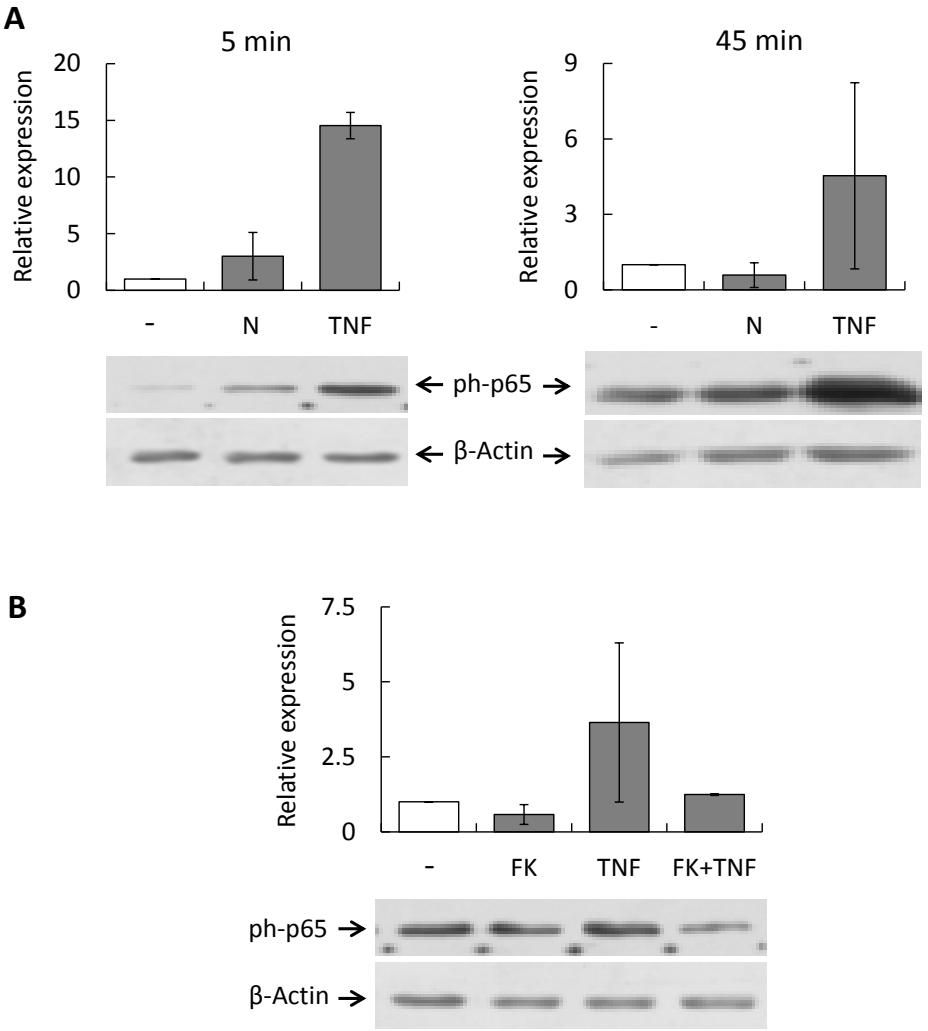

FIGURE 6.2: Effects of NAMPT and NAMPT inhibition on activation of NF-κB in neutrophils A. rhNAMPT (N) induced a small increase in phosphorylation of NF-κB (p65) at 5 min but this was not eveident by 45 min, measured by immunoblotting, **B**. 30 min pre-treatment with FK866 (FK) prevented phosphorylation of p65 in response to TNFα (5 min). Data displayed as relative expression compared to untreated (-), and represents mean ±SEM of 3 independent samples.

6.3.3 Effects of NAMPT and NAMPT inhibition on activation of other key signalling molecules

Due to the effect of NAMPT and NAMPT inhibition on the activation of the NF-κB pathway, it was then investigated whether NAMPT is similarly involved in other key signalling pathways in neutrophils, especially those activated by the cytokine GM-CSF. The effects of NAMPT and FK866 on the neutrophil JAK/STAT and MAPK signalling pathways were investigated by immunoblotting for phosphorylation of the signalling molecules STAT3 and ERK1/2 (p44/p42), respectively. In experiments where GM-CSF (50 U/mL) was used as a positive control for activation of these pathways, rhNAMPT (100 ng/mL) did not induce significant phosphorylation of STAT3 or ERK, although there did appear to be a small increase in phosphorylation of the STAT3 molecule (Fig. 6.3A, B). Next, it was investigated whether inhibition of NAMPT with FK866 could affect the GM-CSF induced phosphorylation of these signalling molecules. Neutrophils were treated for 30 min with FK866 (100 nM) prior to GM-CSF stimulation for 5 min, alongside the appropriate no treatment controls. Pre-incubation with FK866 decreased the GM-CSF induced phosphorylation of both ERK and STAT3 (Fig. 6.3C, D). Phosphorylation of STAT3 was significantly decreased to unstimulated levels when the cells were treated with FK866 prior to GM-CSF stimulation (Fig. 6.3D).

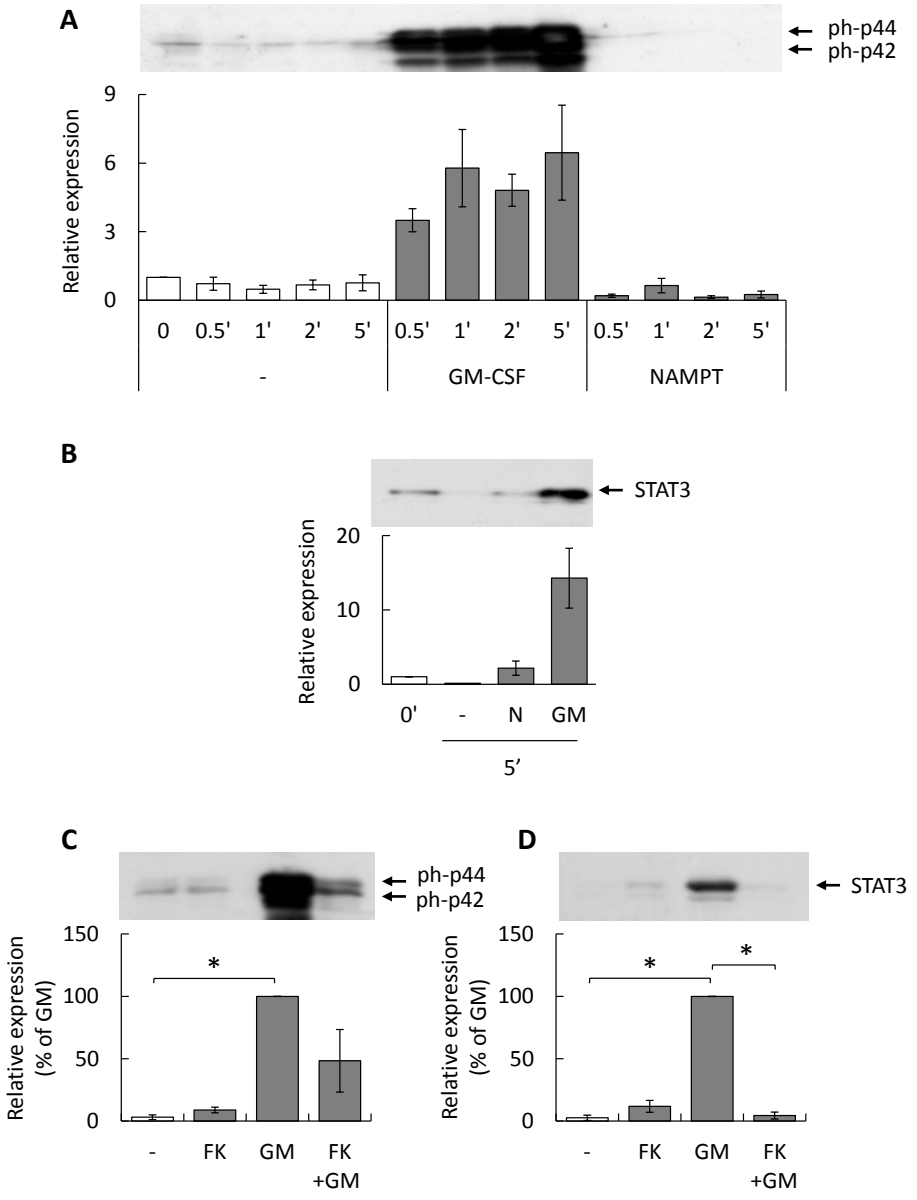

FIGURE 6.3: Effects of NAMPT and NAMPT inhibition on activation of signalling molecules STAT3 and ERK1/2 by immunoblotting. A. GM-CSF induced phosphorylation of ERK1/2 (p44/p42) over 5 min, rhNAMPT (100 ng/mL) did not (n=8). **B.** rhNAMPT (N) induced a slight increase in phosphorylation of STAT3 at 5 min, but this was not to the same magnitude as that induced by GM-CSF (GM) (n=5). Data displayed as relative expression compared to untreated (-) **C.** 30 min pre-incubation of neutrophils with FK866 (FK) (100 nM) decreased the phosphorylation of ERK1/2 and STAT3 induced by GM-CSF (5 min) (n=3). Data displayed as percentage expression of GM-CSF treated group. All data represents mean ±SEM of multiple independent samples as indicated, *$p<0.05$.

6.3.4 Whole transcriptome sequencing to investigate TNFα induced gene expression inhibited by FK866

Having shown that NAMPT inhibition decreases TNFα-induced phosphorylation of NF-κB, and GM-CSF induced phosphorylation of STAT3 and ERK1/2, and also expression of the downstream NF-κB transcriptional target TNFα, it was then investigated whether NAMPT inhibition in neutrophils had an effect on the wider transcriptional profile of neutrophils. Whole transcriptome sequencing of healthy control neutrophils was conducted to compare gene expression between unstimulated cells and those stimulated with TNFα (10 ng/mL) alone, or following a 1 h pre-incubation with the NAMPT inhibitor FK866 (100 nM). RNA was isolated as described previously (2.2.9) from at least 30 x 10^6 neutrophils for each condition, and the RNA integrity number (RIN) was confirmed to be greater than 8 for all samples. Samples were then prepared and sequenced externally using the Illumina HiSeq 2000 Analyser sequencing platform at the Beijing Genomics Institute (BGI). Mapping and annotation of the sequencing data was performed by H. Thomas and H. Wright, as described in 2.2.11. TNFα upregulated many cytokines and transcription factors that are well validated in the literature; for example TNFα, IL-1α and IL-1β, and genes involved in NF-κB signalling. Inhibition of NAMPT for 1 h prior to TNFα stimulation, altered the TNFα induced regulation of many neutrophil genes, and expression levels most changed by addition of FK866, both negatively and positively,

are shown in Tables 6.1 and 6.2 (Appendix), respectively. Of the 17,499 genes identified as being expressed by neutrophils in at least one of the treatment groups, 5046 were down-regulated when cells were pre-incubated with FK866 prior to TNFα stimulation, and the fold decrease from TNFα alone to FK866 plus TNFα was greater than 1.5 in 1310 of these genes (greater than 2 in 402 genes). The numbers of mapped genes in each treatment category, and the change in expression induced by each treatment is summarised in Tables 6.3 and 6.4 below.

Group	Number of mapped genes
Total	17,499
Control	16,389
TNFα	16,238
FK866 + TNFα	16,188

TABLE 6.3: Number of mapped genes in each treatment group.

Change from	Treatment	Change in expression (<1.5 fold)	Number of mapped genes
Control	TNFα	Increase	340
		Decrease	280
	FK866 + TNFα	Increase	864
		Decrease	1569
TNFα	FK866 + TNFα	Increase	1075
		Decrease	1310

TABLE 6.4: Number of genes with altered expression in each treatment group.

The fold difference in expression between TNFα and FK866 plus TNFα was less than 2 for all of the housekeeping genes investigated, and the average fold change across these 5 housekeeping genes was -0.66 (Table 6.5; appendix). It was then investigated whether there was any relationships between the genes whose TNFα induced expression was inhibited by FK866. The software programme Ingenuity Pathway Analysis (IPA) was used to compare the canonical pathways that were statistically most likely to be regulated by the treatment in each sample. Figure 6.4 shows the top 20 pathways predicted to be activated by TNFα stimulation; a number of TNFα receptor pathways are shown, many of which involve NF-κB signalling, which is known to be activated by engagement of TNFα receptors. Figure 6.5 shows the top 20 pathways predicted to be regulated by the change in expression from control cells to those treated with FK866 and TNFα; this shows a very different profile of pathways than those shown to be regulated by TNFα alone. In fact, only 6 of these 20 pathways are common between the two treatments. It is of interest to note that the pathway predicted to be regulated by FK866 pre-treatment with the most confidence is the NRF2-mediated oxidative stress response, which is activated by ROS. It can be seen in Figure 6.6 that expression of many of the participating molecules in this pathway are down-regulated in the samples pre-treated with FK866, as compared to those treated with TNFα alone, consistent with the observation that FK866 inhibits production of ROS by neutrophils.

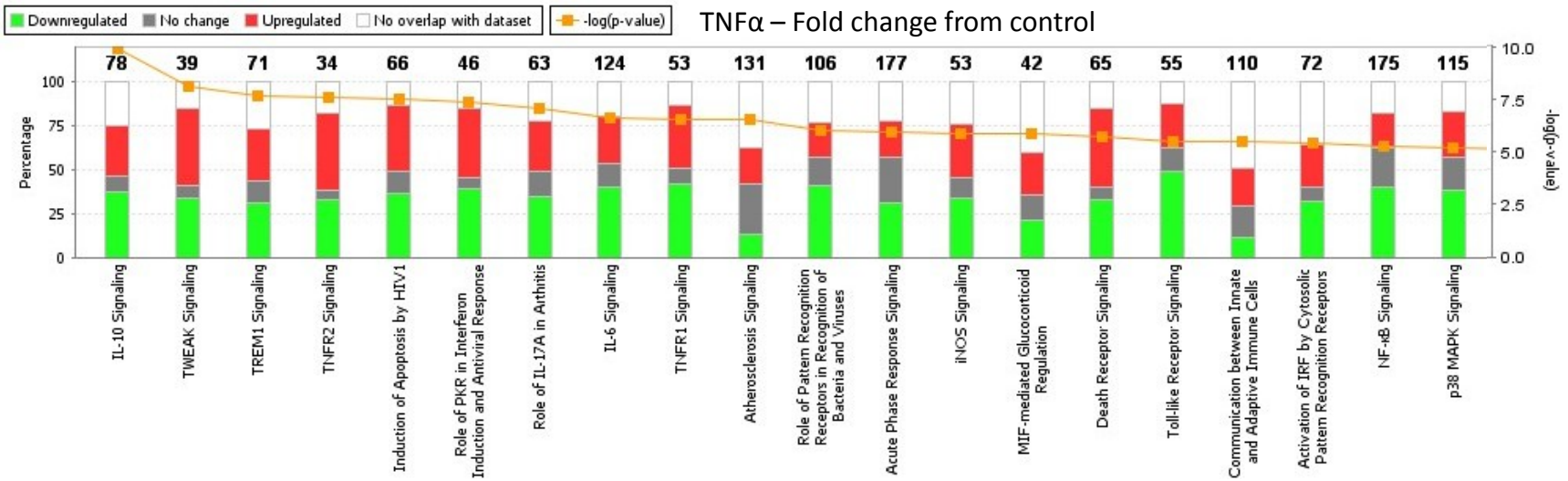

FIGURE 6.4: IPA predicted canonical pathways differentially regulated from control with TNFα or FK866 plus TNFα treatment in neutrophils. From IPA the 20 pathways considered by to be statistically most likely to be differentially regulated from control with TNFα treatment. Genes down-regulated from control - green bar, upregulated – red bar, unchanged - grey and no expression - empty bar. The orange line represents the statistical likelihood (p-value) that the indicated canonical pathway is regulated by the treatment.

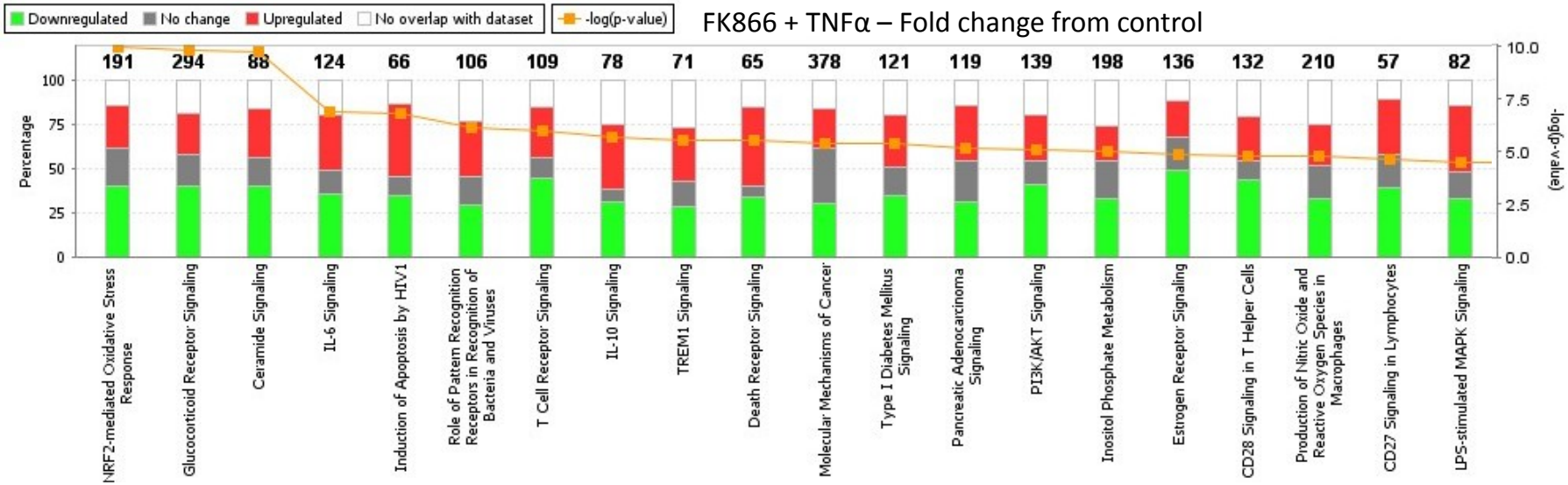

FIGURE 6.5: IPA predicted canonical pathways differentially regulated from control when pre-treated with FK866 prior to TNFα treatment in neutrophils. From IPA the 20 pathways considered by to be statistically most likely to be differentially regulated from control with FK866 plus TNFα treatment. Genes down-regulated from control - green bar, upregulated – red bar, unchanged - grey and no expression - empty bar. The orange line represents the statistical likelihood (p-value) that the indicated canonical pathway is regulated by the treatment.

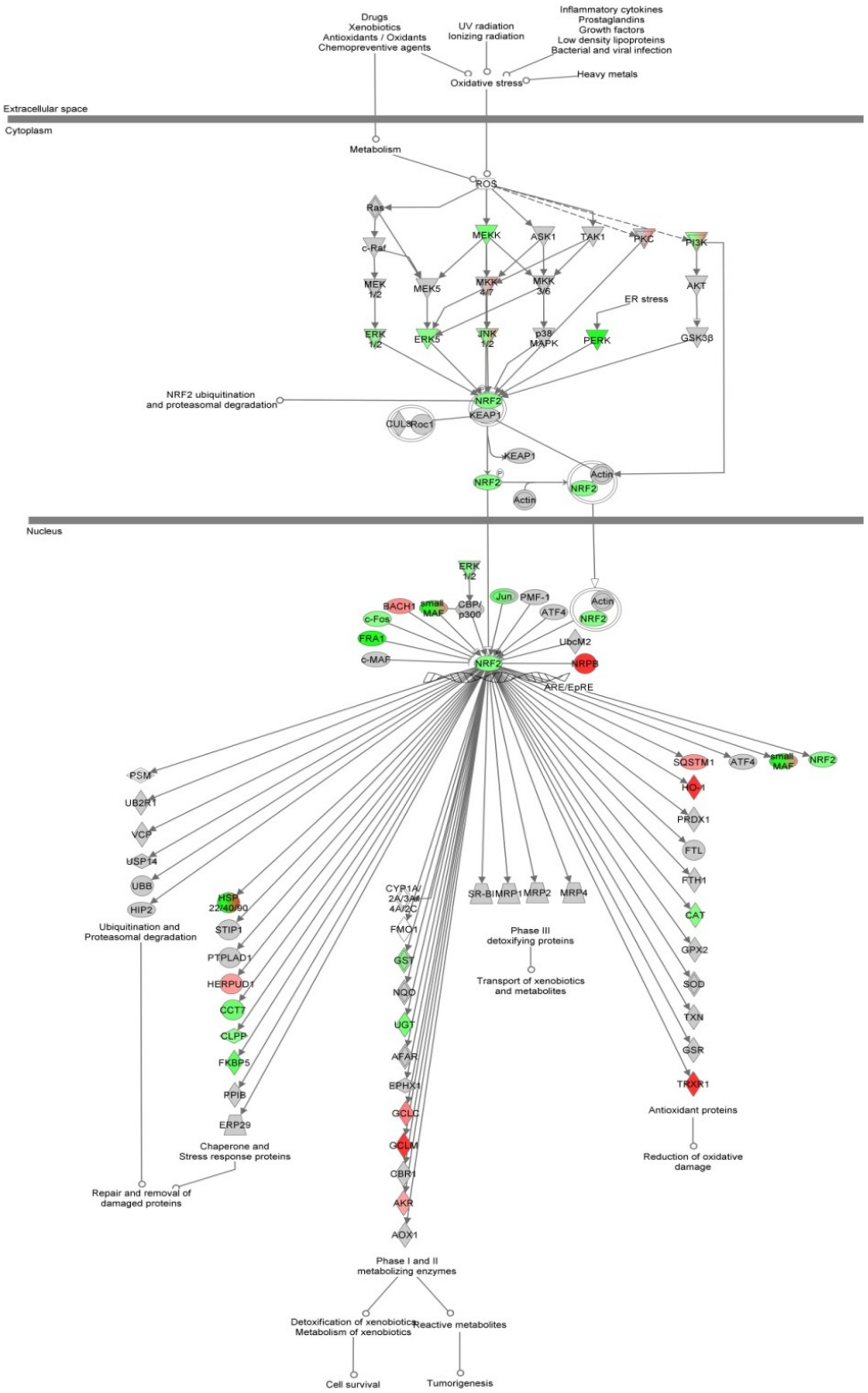

FIGURE 6.6: The NRF2-mediated oxidative stress response pathway is down-regulated by addition of FK866 compared to TNFα signalling alone. This IPA NRF2-mediated stress response pathway is overlaid with the fold changes (>1.5) in expression with FK866 pre-treatment prior to stimulation with TNFα. (change from TNFα alone). Green – decrease; red – increase; grey – no change; white – no expression.

Using IPA it can be predicted which transcription factors are likely to have been affected by each treatment, by comparing the regulation of their downstream targets. Table 6.6 (appendix) shows the 15 transcription factors statistically most likely to be activated by TNFα (as fold change from control), many of these were involved in NF-κB signalling. Of these 15 transcription factors 9 (Table 6.7; appendix) were also predicted by IPA as being statistically most likely to be down-regulated by FK866 pre-treatment prior to TNFα stimulation (as fold change from TNFα alone); again many of these were involved in the NF-κB pathway. Similarly, 6 transcription factors were predicted by IPA to be down-regulated from control by TNFα treatment, and 3 of these were also predicted with most certainty to be upregulated when cells were pre-treated with FK866 (Tables 6.8 and 6.9; appendix). Taken together, this preliminary analysis of the data suggests that pre-incubation with FK866 abrogates much of the TNFα induced gene expression in neutrophils.

The differential expression of genes involved in the NF-κB pathway was explored in more detail alongside genes involved in neutrophil apoptosis, the NADPH oxidase complex and those coding for inflammatory cytokines and chemokines, based on the previously observed effects of NAMPT inhibition on neutrophil functions.

6.3.5 Effects of NAMPT inhibition on the TNFα induced expression of components of the NF-κB pathway in neutrophils

TNFα stimulated expression of many of the genes encoding components of the NF-κB pathway, but especially the inhibitors of NF-κB (IκB) and A20

(TNFAIP3), the product of which is responsible for the termination of TNFα induced activation of NF-κB. However, some genes involved in TNFα signalling of neutrophils were down-regulated following TNFα stimulation, including the TNF receptor 1 (TNFRSF1A), FADD, and the components of the NF-κB specific MAP kinase complex, NIK/MEKK1 (Fig. 6.7). This represents the TNFα/NF-κB negative feedback response. Pre-incubation with the NAMPT inhibitor prior to TNFα stimulation resulted in >2-fold decreased transcripts for 10 out of the 26 NF-κB related genes, when compared to TNFα stimulation alone (Fig. 6.7). A summary of the change in expression of genes involved in NF-κB signalling is shown in Figure 6.8; 6.8A shows that many elements of the pathway are upregulated (red) by TNFα stimulation in contrast to 6.8B where many of the genes are down-regulated (green) compared to TNFα when samples were first pre-treated with FK866 prior to stimulation.

6.3.6 Effects of NAMPT inhibition on the TNFα induced expression of cytokines and chemokines from neutrophils

It was also investigated whether NAMPT inhibition affected events downstream of signalling pathway activation, by examining the effect of FK866 on expression of inflammatory cytokines. TNFα is known to stimulate cytokine expression from neutrophils, but pre-treatment with FK866 abrogated the TNFα induced upregulation of the transcripts for a number of pro-inflammatory cytokines and chemokines including IL-1α, IL-1β, IL-1 receptor agonist (RA), CCL3 (MIP1α), CCL4 (MIP1β), CXCL2 (GROβ) and TNFα (Fig. 6.9). A summary of the effects of

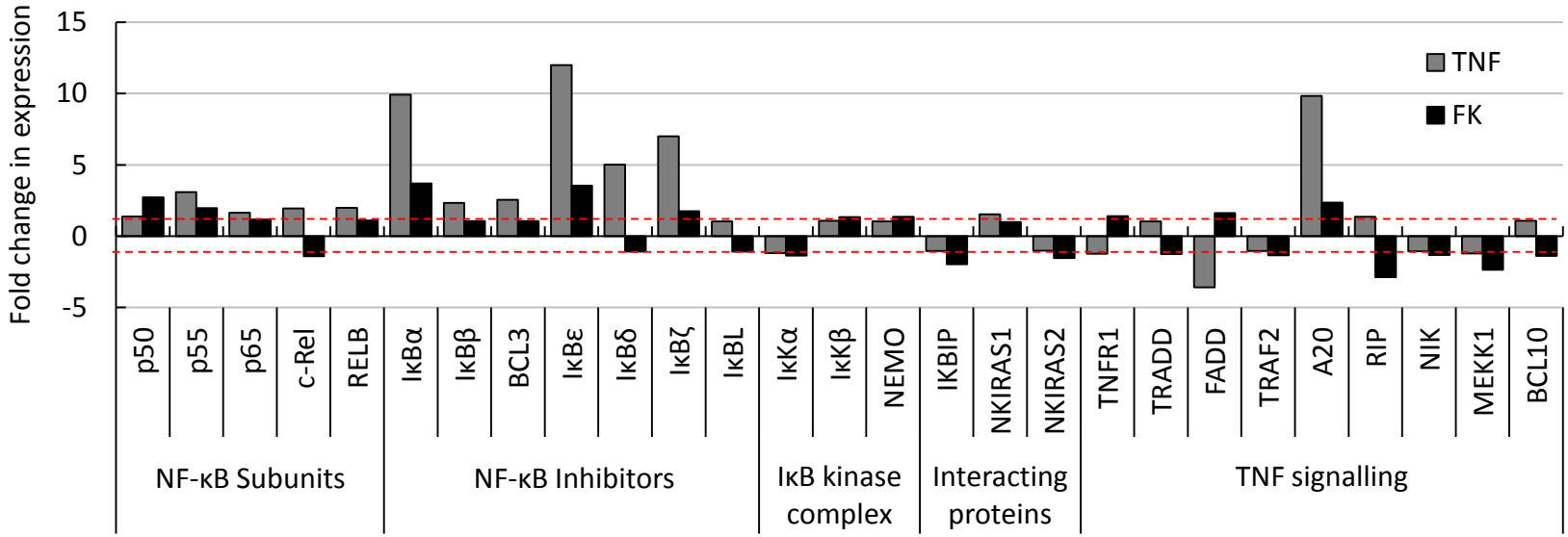

FIGURE 6.7: Genes differentially expressed in the NF-κB pathway between neutrophils stimulated with TNFα alone or those pre-incubated with FK866 prior to TNFα stimulation. The fold change in expression of key components of the NF-κB signalling pathway from control (untreated) cells, to those either treated with TNFα alone (TNF – grey bars) or pre-treated with FK866 prior to TNFα stimulation (FK – black bars). Numbers >1 indicate a change in expression (red line). FK866 decreases the TNFα induced expression of a number of NF-κB pathway genes.

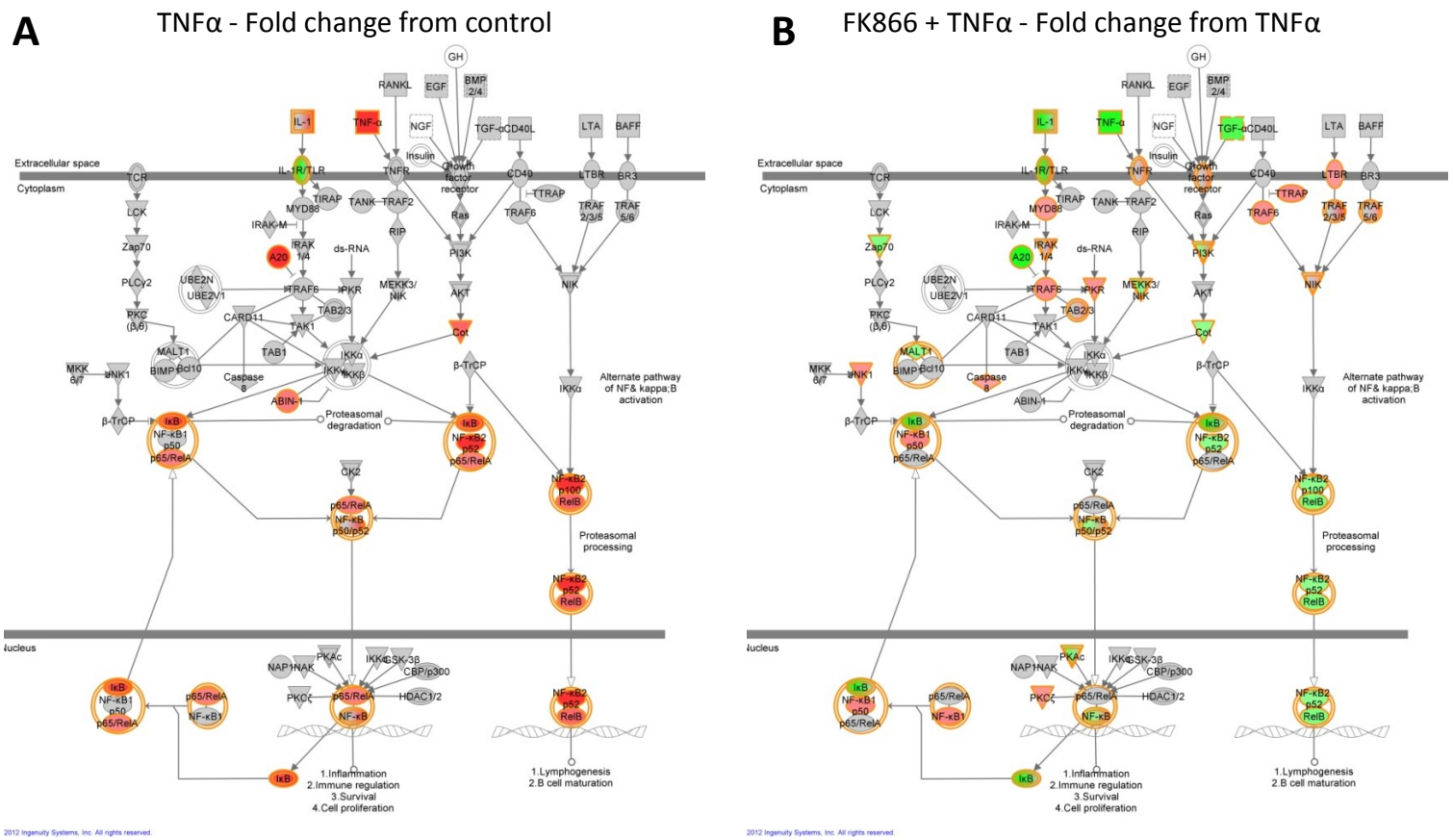

FIGURE 6.8: Genes differentially expressed in the NF-κB pathway between neutrophils stimulated with TNFα alone or those pre-incubated with FK866 prior to stimulation. NF-κB pathway from IPA is overlaid with the fold changes (<1.5 fold) induced by **A.** TNFα (fold change from control) or **B.** FK866 pre-treatment plus TNFα (fold change from TNFα alone). Green – down-regulated, red – upregulated, grey – no change, white – no expression

NAMPT inhibition prior to TNFα stimulation on cytokine and chemokine signalling networks is shown in Figure 6.10.

6.3.7 Effects of NAMPT inhibition on the TNFα induced expression of regulators of apoptosis from neutrophils

FK866 was developed as a pro-apoptotic agent, although no effects on neutrophil apoptosis were seen in this study (Chapter 4). Here, the effects of this inhibitor in combination with TNFα on the expression of apoptosis-related genes were investigated, to establish whether the effects on gene expression indicated a pro-apoptotic effect. At the concentration used in this study (10 ng/mL), TNFα is anti-apoptotic for neutrophils, although at higher concentrations (>10 ng/mL) it can induce apoptosis [133]. TNFα stimulation had a range of effects on the expression of both pro- and anti-apoptotic genes. However, it did induce down-regulation of expression of over half of the TNFα death receptor domain encoding genes expressed (CRADD, DAPK1, FADD, TNFRSF1A and TNFRSF1B). Pre-incubation with FK866 prior to TNFα stimulation led to an increase in death receptor expression in 6 out of the 9 genes investigated (Fig. 6.11A). The products of the genes whose TNF stimulated expression was most changed by addition of FK866 exert both pro-and anti-apoptotic affects. Additionally, a >2fold change in expression of 34/64 apoptosis-related genes was observed following incubation with FK866 prior to TNFα stimulation, compared to TNFα alone (Fig. 6.11A-C). Inhibition of NAMPT appeared to have an

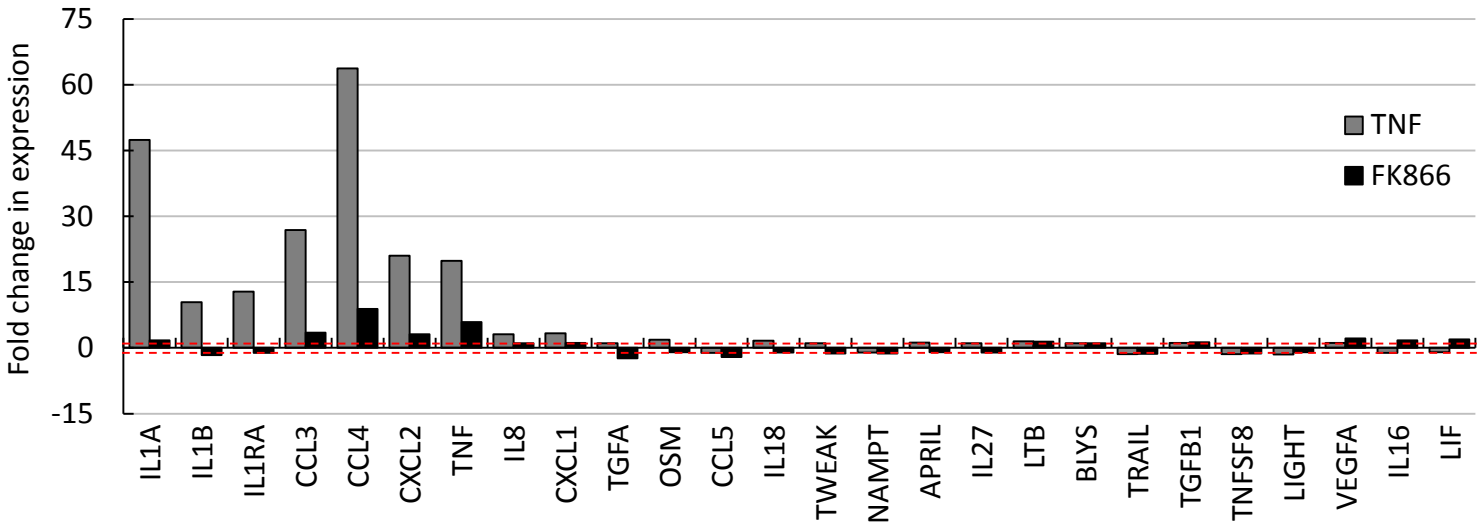

FIGURE 6.9: The effects of NAMPT inhibition on the TNFα induced expression of genes encoding cytokines and chemokines in neutrophils. The fold change in expression of key neutrophil cytokines and chemokines from control (untreated) cells, to those either treated with TNFα alone (TNF – grey bars) or pre-treated with FK866 prior to TNFα stimulation (FK866 – black bars). Numbers >1 indicate a change in expression (red line). FK866 decreases the TNFα induced expression of a number of genes encoding cytokines and chemokines.

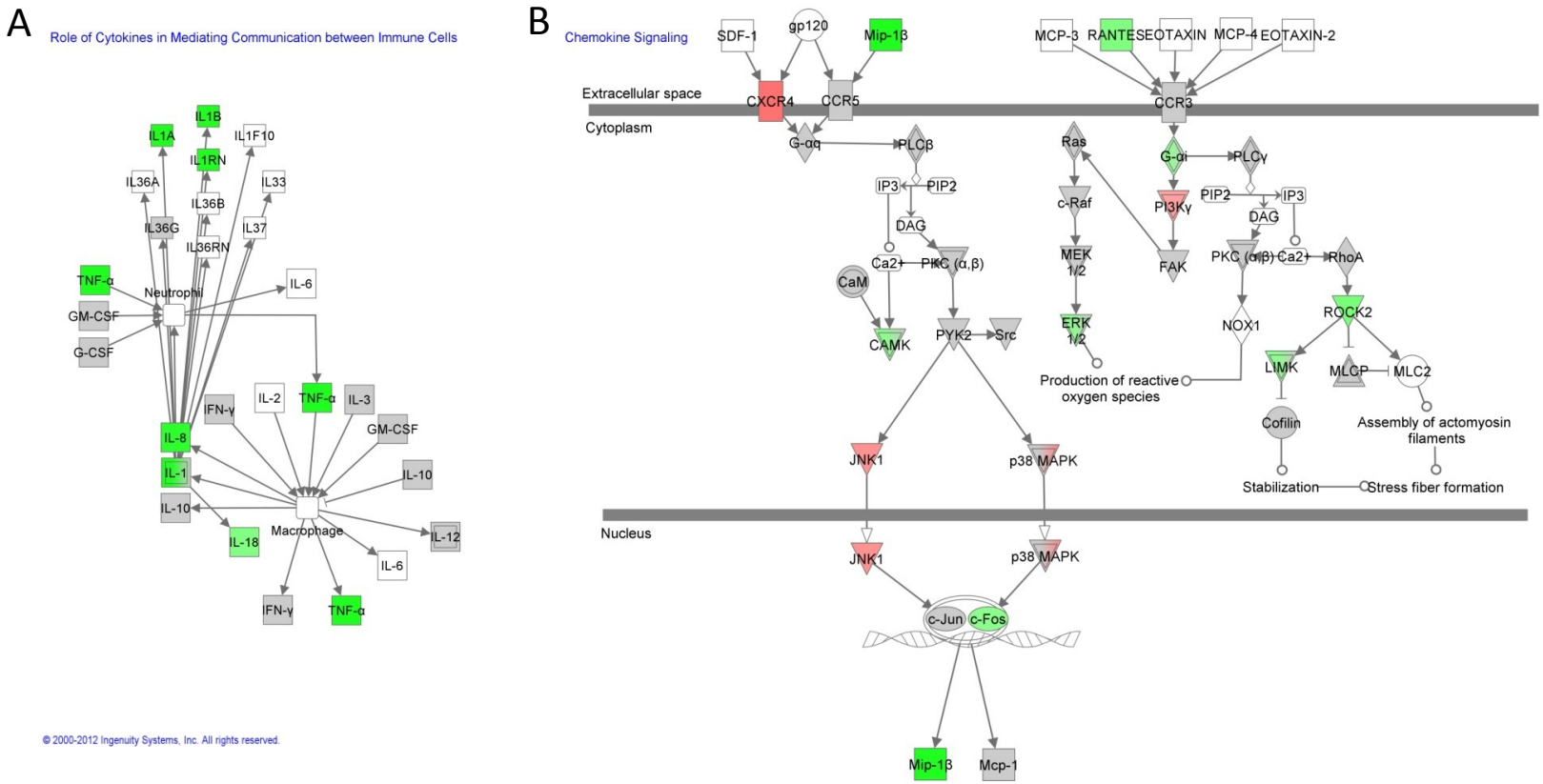

FIGURE 6.10: NAMPT inhibition decreaes the TNFα induced activation of cytokine and chemokine pathways. Key neutrophil cytokines and chemokines (**A.**) and the canonical chemokine signalling pathway (**B.**) from IPA were overlaid with the fold change (<1.5 fold) induced by FK866 pre-treatment plus TNFα (fold change from TNFα alone). Green – down-regulated, red – upregulated, grey – no change, white – no expression.

overall pro-apoptotic effect on the expression of the Bcl-2 family members. The Bcl-2 family of proteins mediate the intrinsic apoptotic pathway by controlling release of apoptotic agents from the mitochondria. This group can be further divided into subfamilies with pro- and anti-apoptotic action. Pre-incubation with FK866 reversed the TNFα induced increase in expression of anti-apoptotic Bcl-2 subfamily members MCL1 and A1 to below control levels, and it also reversed the TNFα induced decrease in expression of pro-apoptotic BH3 subfamily members BIK, BINP3 and BIML, increasing their expression above control (Fig. 6.11B). Figure 6.12 shows a comparison of the genes expressed in the apoptotic signalling pathways when cells were stimulated with TNFα or FK866 and TNFα in combination. FK866 appeared to cause an overall down-regulation of expression, especially on members of the NF-κB signalling pathway as discussed previously. However, NAMPT inhibition increased expression of the pro-apoptotic regulators BIM and BAX, whilst it decreased expression of anti-apoptotic regulators such as members of the cIAP (cellular inhibitor of apoptosis) complex and Mcl-1.

6.3.8 Effects of NAMPT inhibition on the TNFα induced expression of components of the neutrophil NADPH oxidase complex

Due to the significant inhibition of the neutrophil respiratory burst with NAMPT inhibition, the effects of FK866 on the expression of transcripts coding for components of the NADPH oxidase and related proteins were also examined. The core NADPH oxidase consists of the membrane bound $p22^{PHOX}$ (CYBA) and $gp91^{PHOX}$ (CYBB) subunits that form cytochrome

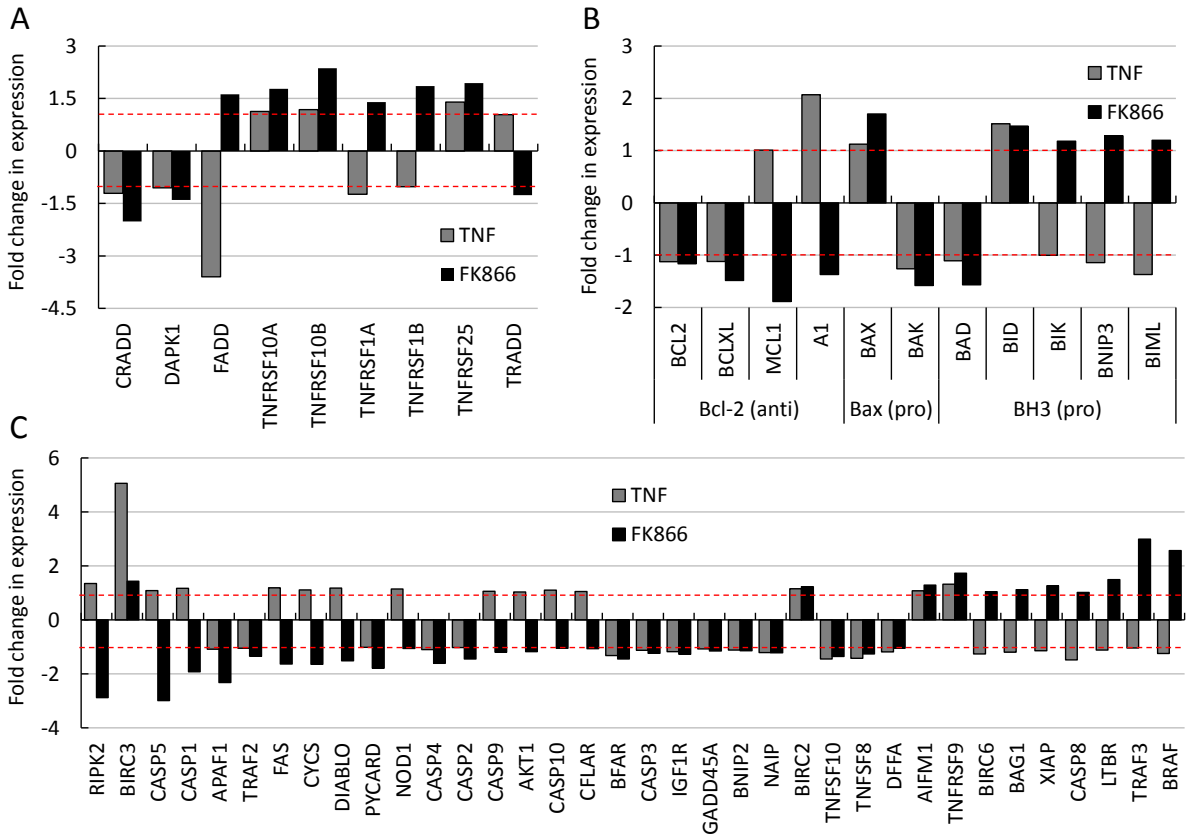

FIGURE 6.11: The effects of TNFα and FK866 pre-treatment on the expression of genes involved in the regulation of apoptosis in neutrophils. The fold change in expression of death receptor (**A.**), Bcl-2 family (**B.**) and other apoptosis regulating genes (**C.**) is shown from control (untreated) cells, to those either treated with TNFα alone (TNF – grey bars) or pre-treated with FK866 prior to TNFα stimulation (FK866 – black bars). Numbers >1 indicate a change in expression (red line). FK866 altered the TNFα induced expression of a number of genes in the Bcl-2 family representing a more pro-apoptotic profile (**B.**)

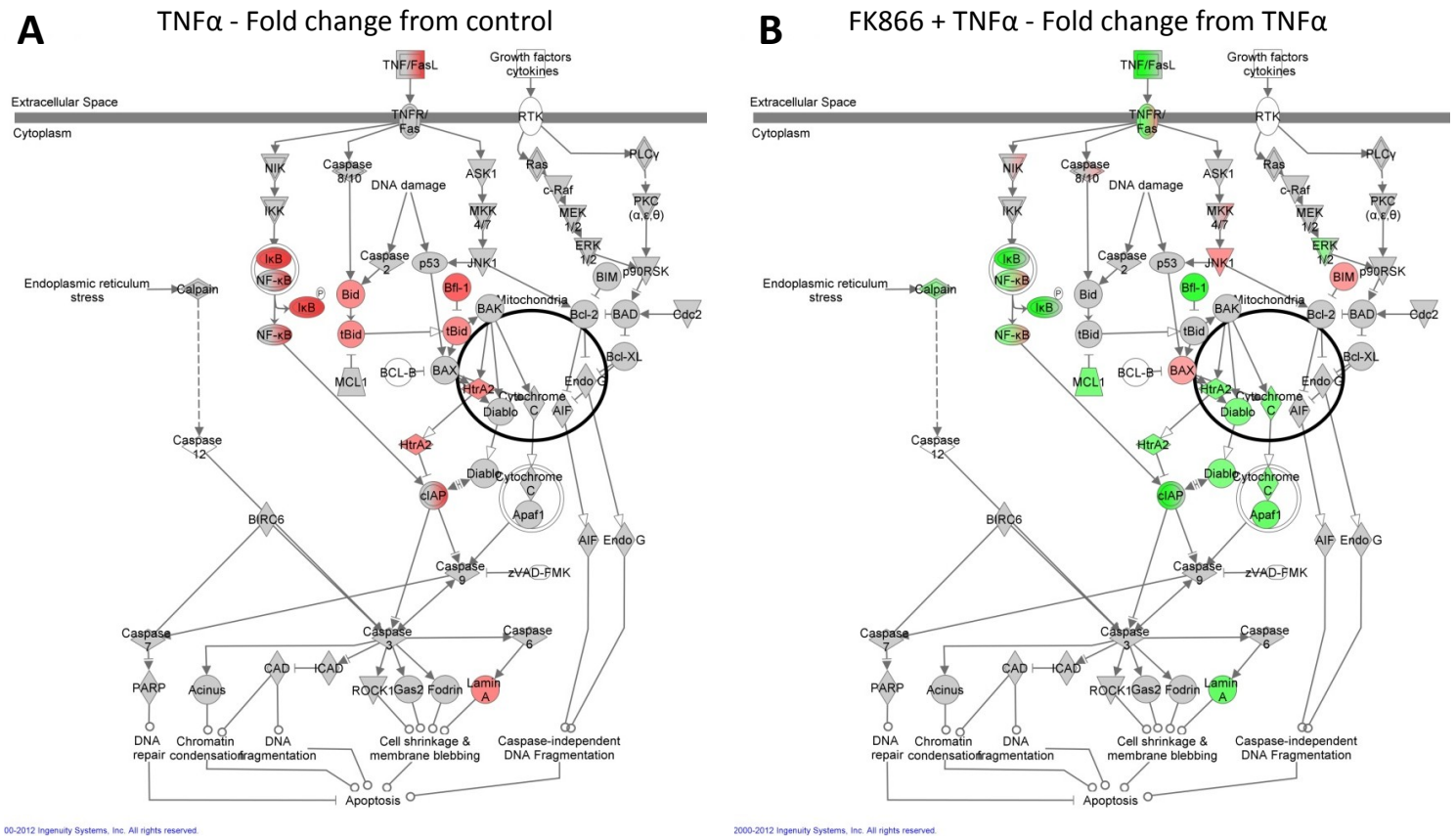

FIGURE 6.12: The effects of NAMPT inhibition on the TNFα induced activation of apoptotic pathways. The apoptotic signalling pathway from IPA was overlaid with the fold changes (<1.5 fold) induced by **A.** TNFα (fold change from control) or **B.** FK866 pre-treatment plus TNFα (fold change from TNFα alone). Green – down-regulated, red – upregulated, grey – no change, white – no expression.

b_{558}, and the cytosolic components p40 ^{PHOX} (NCF4), p47 ^{PHOX} (NCF1), p67 ^{PHOX} (NCF2). Stimulation with TNFα appeared to slightly increase transcripts for many components of the oxidase, but this was less than 1.5-fold in all cases. TNFα also decreased expression of p67 ^{PHOX} 1.6-fold (Fig. 6.13A). Pre-treatment with FK866 down-regulated the TNFα induced transcripts in many of the oxidase components, although again these changes were no greater than 1.5-fold in any component. This is summarised in Figure 6.13B. FK866 pre-treatment had no effect on the expression of the transcripts for the oxidase-associated proteins cytoplasmic Rac2 and membrane bound Rap1A that bind to the core complex. However, FK866 did slightly decrease the TNFα induced expression of RhoGPIα and RhoGPIβ (1.5 and 1.3-fold respectively), that both serve to sequester Rac2 in the cytoplasm and prevent binding to the core oxidase complex (Fig. 6.13A).

6.4 Discussion

The role of NAMPT as a cytokine in the absence of its enzyme activity remains controversial, and many studies do not address the role of NAMPT in NAD synthesis when investigating its cytokine-like function. I have shown here that exogenous NAMPT has some stimulatory effect on the phosphorylation of signalling molecules NF-κB and STAT3, but these changes were much lower than those stimulated by the inflammatory cytokines, TNFα and GM-CSF. These low-level

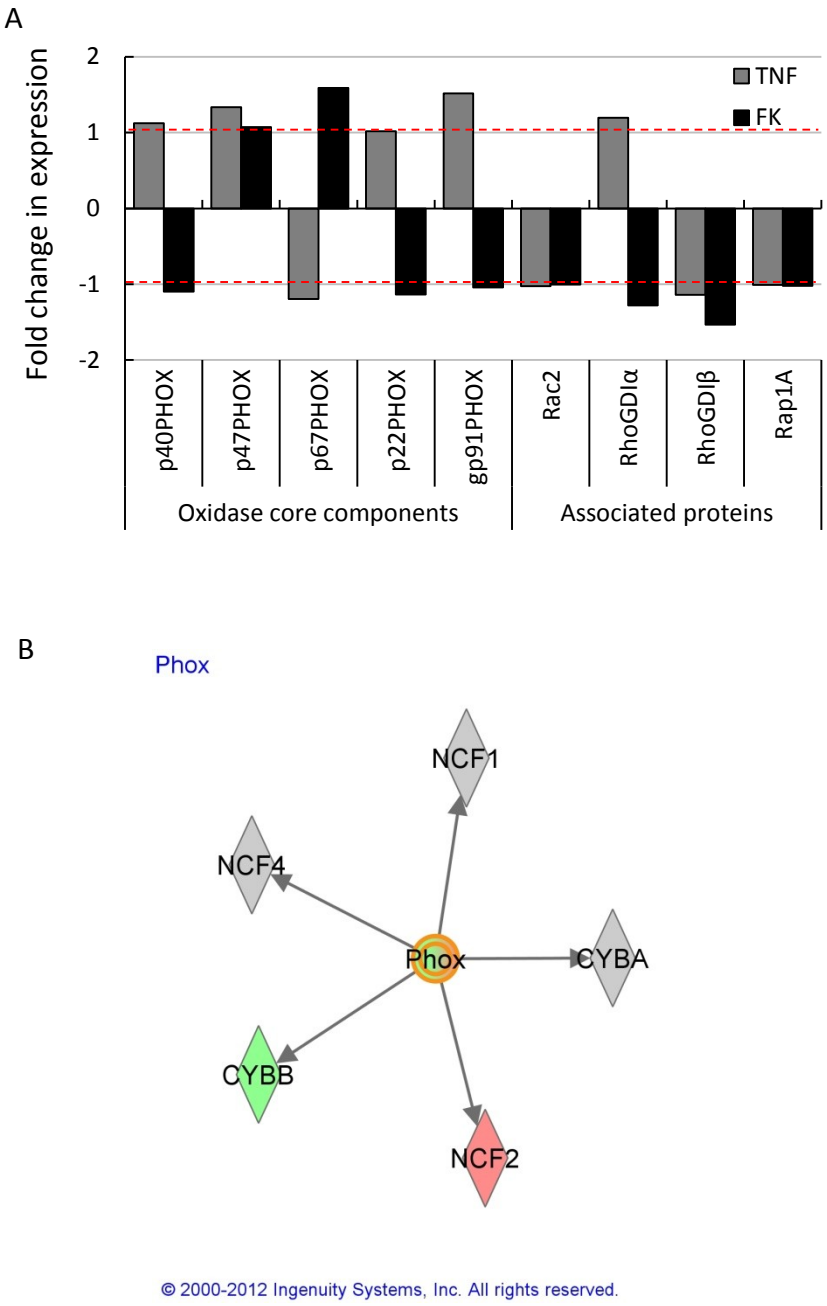

FIGURE 6.13: The effects of TNF and FK866 and TNF in combination on the expression of genes coding for compnents of the NADPH oxidase. A. Shown is the fold change in expression of neutrophil oxidase components from control (untreated) cells, to those either treated with TNFα alone (TNF – grey bars) or pre-treated with FK866 prior to TNFα stimulation (FK866 – black bars). Numbers >1 indicate a change in expression (red line) **B.** Interacting components of the oxidase from IPA was overlaid with the fold changes (<1.5 fold) induced by FK866 pre-treatment plus TNFα (fold change from TNFα alone). Green – down-regulated, red – upregulated, grey – no change, white – no expression.

stimulatory effects were not paralleled by changes in the transcription of cytokines regulated by these signalling pathways. In support of these low level effects, addition of NAMPT also had little effect on neutrophil function, as detailed in Chapter 4, although exogenous NAMPT did delay neutrophil apoptosis and the turnover of Mcl-1, as described in section 4.3.4. These experiments appear to indicate that the function of NAMPT as a cytokine-like signalling molecule with neutrophils is rather limited to its affect on apoptosis, although this could be further validated by simultaneously blocking NAMPT enzyme activity. There has been more convincing evidence of NAMPT functioning as a cytokine reported for other cell types. For example, NAMPT in the absence of its enzyme activity, has been shown to stimulate cytokine expression and delay apoptosis in endothelial cells and macrophages respectively [21,22].

It is clear that NAMPT exerts a number of effects on neutrophils when acting as an enzyme. Experiments inhibiting the function of the NAMPT enzyme in neutrophils show that this inhibition abrogated activation of signalling molecules NF-κB, STAT3 and ERK1/2 that regulate key neutrophil signalling pathways controlling a wide variety of neutrophil processes including development, function, cytokine expression and apoptosis [267–269]. This is seen also in the wide-ranging effects of NAMPT inhibition on the neutrophil transcriptional profile; neutrophils pre-treated with FK866 showed many differentially regulated genes when stimulated

with TNFα, compared to those not pre-treated with the inhibitor. However, these effects were not global, as the expression of less than 15% of the genes mapped was altered by NAMPT inhibition, when compared to untreated or TNFα treated cells, suggesting specific targeted effects. Some of the greatest effects on expression were seen on those genes and pathways downstream of NF-κB activation, as TNFα stimulation alone induces significant activation of this pathway, which is abrogated when NAMPT is inhibited. As might be expected from inhibition of this pathway, NAMPT inhibition also decreases the TNFα-induced expression of a number a key inflammatory cytokines and chemokines. *In vivo* a decrease in the inflammatory cytokines released by neutrophils would be expected to dampen the inflammatory response and could be beneficial in inflammatory conditions where the level of inflammation is inappropriate. A number of experiments using FK866 in animal models of inflammatory joint disease have shown a decrease in inflammatory cytokines such as TNFα in the circulation, and a corresponding improvement in joint swelling and destruction [33,34]. NAMPT inhibition also affected the expression of a number genes involved in control of neutrophil apoptosis. It is important that neutrophil apoptosis is regulated to enable an appropriate response to immune challenge, but to also to prevent inappropriate release of potentially tissue-damaging products from neutrophils. Dysregulated neutrophil apoptosis is a feature of many inflammatory diseases such as RA [11].

FK866 was developed as a pro-apoptotic agent that induced apoptosis via the intrinsic (mitochondrial) pathway following depletion of NAD over a number of days [206]. However, NAMPT inhibition had no effect on neutrophil apoptosis measured in this thesis by annexin V binding and uptake of PI (section 3.3.6). As neutrophils have a relatively short lifespan (estimated at 6-18 h [38]), the pro-apoptotic effects of FK866 may be masked by the high rates of constitutive neutrophil apoptosis. However, when neutrophils are pre-treated with FK866 prior to stimulation with an anti-apoptotic concentration of TNFα, the transcriptional profile changes dramatically from cells stimulated with TNFα alone. NAMPT inhibition altered expression of both pro- and anti-apoptotic regulators, but FK866 pre-treated cells exhibited a generally pro-apoptotic transcriptional profile with regard to members of the Bcl-2 family. Most notably, FK866 pre-treatment prior to TNFα stimulation caused down-regulation of expression of anti-apoptotic Mcl-1 and upregulation of the pro-apoptotic BIM and BAX. Turnover of Mcl-1 is the major way in which neutrophils regulate apoptosis, as this pro-survival protein has a short half-life of around 2 h, and caspase-mediated turnover facilitates constitutive neutrophil apoptosis in the absence of stimulus. Pro-inflammatory stimuli alter the phosphorylation state of Mcl-1 to delay its cleavage and promote neutrophil survival, but they do not generally induce more than a small increase in Mcl-1 mRNA expression [38,133], as is shown here with TNFα stimulation. NAMPT

inhibition prior to TNFα addition, however, decreases transcript levels for Mcl-1, and this alongside the upregulation of pro-apoptotic Bcl-2 family members BIM and BAX, demonstrates that FK866 inhibits the anti-apoptotic action of low dose TNFα on neutrophils.

It was not within the scope of this experiment to explain the mechanism for these effects on the neutrophil transcription, although it has been shown previously that FK866 can affect transcription by decreasing the availability of NAD for NAD-consuming enzymes involved in transcriptional control, such as sirtuins [33,35,169]. However, I have also shown previously that inhibition of NAMPT causes a significant decrease in the amount of ROS produced by neutrophils (Chapter 3). In addition to their role in bacterial killing, ROS can also act as signalling molecules [270]. It may be that the inhibition of ROS production by FK866 has downstream effects on ROS-mediated signalling pathways, as indicated by the large-scale down-regulation of the NRF2-mediated oxidative stress response pathway predicted by IPA, which is activated by ROS.

This effect of NAMPT inhibition on the activity of the ROS-generating NADPH oxidase complex was also investigated here. It was hypothesised that FK866 inhibited production of ROS by depleting the NAD pool and decreasing the availability of NADP that provides the reducing power for the oxidase. The results shown here provide no evidence that the decrease in ROS is due to decreased expression of the oxidant components or its

associated regulatory proteins, so the inhibition is likely to be linked to the NAD bioavailability.

The whole transcriptome analysis of NAMPT-inhibited neutrophils is a powerful tool that provides a large amount of information about the role of NAMPT and NAD in the neutrophil. The function of this enzyme is clearly important for a number of signalling pathways, production of inflammatory mediators and control of apoptosis, and it can be appreciated how changes in these processes could affect the neutrophil functions studied in Chapter 3. Most of these observed effects can be attributed to depletion of NAD in the cell, but these preliminary studies suggest that a more detailed investigation into the mechanisms responsible for this down-regulation of gene expression is warranted. However, as the effects of NAMPT inhibition resulted in a selective decrease in gene expression, it may be predicted that more-specific mechanisms, other than NAD depletion, are responsible. Although this transcriptomic sequencing has only been carried out once to date, and will require further validation, the correspondence with previous findings in this thesis and in the wider literature, endorses the validity of the data.

CHAPTER 7: General Discussion and Conclusions

Neutrophils have a pathologically important role in the progression of chronic inflammatory diseases such as rheumatoid arthritis, and have a great capacity to cause tissue damage by virtue of their cytotoxic contents. This research aimed to examine the role of the pleiotropic protein NAMPT in the regulation of neutrophil functions that are important in inflammation, including the rate of neutrophil apoptosis and expression of mRNA from these cells. The role of NAMPT, as both an NAD-biosynthesis enzyme and as a cytokine-like molecule has been evaluated; the most striking effects on neutrophil function and mRNA expression have been mediated by inhibiting NAMPT enzyme.

The main findings in this thesis were as follows:

- Inhibition of NAMPT depleted intracellular NAD(P)H, and decreased the ability of neutrophils to produce reactive oxidants in response to both receptor-mediated and receptor-independent stimulation. Despite this, NAMPT inhibition did not affect phagocytosis or bacterial killing (Chapter 3).
- Exogenous NAMPT delayed constitutive neutrophil apoptosis by stabilising the key neutrophil anti-apoptotic protein Mcl-1. NAMPT inhibition had no effect on this process (Chapters 3 and 4).

- NAMPT was constitutively expressed by neutrophils but did not appear to be dynamically regulated by cytokines; however, NAMPT expression was elevated in synovial fluid neutrophils of RA patients compared to paired blood neutrophils. NAMPT mRNA expression also appeared to correlate with expression of TNFα in RA patients considered for biologic therapy, prior to starting treatment (Chapter 5).
- NAMPT inhibition decreased the activation of a number of signalling molecules (NF-κB, ERK, STAT3) in response to stimulation of neutrophils, and decreased the expression of TNFα in response to stimuli (Chapter 6).
- NAMPT inhibition also had wide-ranging effects on neutrophil gene expression. RNA sequencing identified that FK866 pre-treatment decreased the TNF-induced expression of a number of genes, including those encoding cytokines and chemokines. NAMPT inhibition prior to TNFα stimulation led to a distinct change in the overall transcriptional profile compared to neutrophils stimulated with TNFα alone. However, NAMPT inhibition resulted in selective, rather than global, changes in mRNA levels (Chapter 6).

In Chapter 3, the effects of NAMPT inhibition on neutrophil functions central to roles in both innate immunity and chronic inflammation were

investigated. *In vivo* a number of mechanisms act in concert to facilitate neutrophil activation and response to inflammatory stimuli. In this Chapter, these processes were investigated in isolation to determine the effects of NAMPT inhibition and NAD depletion on each of these stages. Initial experiments identified that FK866 depleted intracellular NAD/H and NADP/H, and that pre-incubation of freshly isolated neutrophils with FK866 prevented the increases in NAD(P)/H observed during priming and activation. Neutrophils were pre-incubated with FK866 for at least 30 min in subsequent experiments, as preliminary experiments identified that NAMPT inhibition had the greatest effect when time was allowed for NAD(P)H depletion to occur prior to stimulation of function. Where possible neutrophils were pre-treated for 1 h, however for many functional tests (especially those that relied on membrane receptor expression), control neutrophils became less responsive after a >30 min pre-incubation time. The prominent results from this Chapter were that NAMPT inhibition decreased the ability of neutrophils to mount a respiratory burst in response to both receptor-independent (PMA) and receptor-dependent stimuli, which included both bacterial (fMLP and *S. aureus*) and inflammatory stimuli (immune complexes); however this decrease in the amount of ROS produced by NAMPT-inhibited neutrophils did not decrease the capacity for bacterial killing. Precedent for this has been shown in some patients with autosomal recessive CGD who are able to maintain sufficient bacterial killing despite defects in the NADPH oxidase [100]. It was

hypothesised that FK866 decreased the amount of ROS produced by neutrophils because it decreased the availability of NADPH required to fuel reduction of molecular oxygen in the first step of the respiratory burst. It was confirmed that FK866 inhibited production of the initial oxygen free radical, O_2^-, and inhibited the increase of NADP/H stimulated by PMA. It has been shown previously that the neutrophil intracellular concentration of NADP/H is increased greater than 3-fold upon activation, to provide a sufficient store of reducing power to the oxidase [83]. The NADPH oxidase is a multi-component enzyme made up of pre-formed subunits that are assembled at the plasma membrane upon neutrophil priming. The fact that the amount of ROS produced by neutrophils was decreased in a time- and dose-dependent manner by NAMPT inhibition, rather than completely ablated, lends credence to the hypothesis that inhibition occurs due to the gradual depletion of NADPH. Also, in Chapter 6 it was shown that NAMPT inhibition did not have any effect on the *de novo* transcription of the oxidase subunits. It would be of interest to further investigate the contribution of NAD metabolism to neutrophil ROS production by inhibiting other enzymes of the NAD biosynthetic pathway, such as NMNAT and NAD kinase. The decrease in production of ROS by neutrophils achieved after NAMPT inhibition, without a concomitant decrease in the capacity for bacterial killing, suggest that FK866 could be a viable therapeutic agent to modulate the destructive capacity of neutrophils in inflammatory disease, without adversely compromising host defence. In

support of this, animal models of inflammatory arthritis have shown improvement following administration of FK866, which included a decrease in radiological joint damage, and decrease in circulating cytokines [33,34]. One study suggested that inhibition of NAMPT is as effective as targeting of TNFα by etanercept in these models [33].

This same approach to investigating the effects of NAMPT inhibition on neutrophil function was also employed to assess the functional effects of stimulation with exogenous NAMPT, with the aim of addressing whether NAMPT could activate neutrophils like a cytokine, in the absence of an identifiable NAMPT surface receptor. NAMPT is elevated in inflammation, and NAMPT serum concentration correlates with disease activity in RA [18,32,165,218]. It is proposed that exogenous NAMPT can stimulate the activity of immune cells, such as macrophages [22] and it has been reported to exert pro-inflammatory effects on neutrophils; NAMPT delays neutrophil apoptosis and contributes to assembly of the NADPH oxidase [19,224]. For the majority of neutrophil functions tested in this thesis, exogenous recombinant NAMPT at pathophysiological concentrations did not induce a primed phenotype, as compared to treatment with known priming agents such as GM-CSF and TNFα. This was with the exception of the effect of NAMPT on neutrophil apoptosis; in line with other published data [19], NAMPT significantly delayed neutrophil apoptosis, and this was achieved by stabilisation of the anti-apoptotic protein, Mcl-1.

NAMPT inhibition had no effect on neutrophil apoptosis and turnover of Mcl-1. This was initially surprising as FK866 was developed as a pro-apoptotic anti-cancer agent, but its effect on cell line apoptosis manifests over a number of days, as NAD is gradually depleted leading to eventual cell death via the intrinsic apoptotic pathway [205,206]. However, due to the short half-life of neutrophils, this effect was likely masked by constitutive apoptosis. It has been proposed that NAMPT decreases the activity of caspases-8 and -3 in neutrophils, but not caspase-9, suggesting that NAMPT inhibits the extrinsic or death-receptor mediated apoptotic pathway [19]. Whether exogenous NAMPT directly delays apoptosis, or whether it occurs in an autocrine/paracrine manner via activation of expression of other cytokines, is not clear from this research. However, in Chapter 7 of this thesis, it was shown that although NAMPT may stimulate low levels of activation of NF-κB and STAT3, it does not appear to robustly induce downstream expression of the inflammatory mediators that were examined. It would be necessary to investigate the effects of NAMPT on neutrophils, in combination with other inflammatory cytokines, and with other immune cell types, as this would be more indicative of the inflammatory environment *in vivo*. It may be that NAMPT has a synergistic effect with other cytokines, as it does with IL-17 on pre-B-cell maturation [167], or that the NAMPT-stimulated expression of inflammatory mediators from other cell types indirectly leads to enhanced neutrophil activation.

NAMPT is present at relatively high concentrations at sites of chronic inflammation, such as in the rheumatoid joint [18,32,165], and it has been reported that serum NAMPT correlates with leukocyte counts in children [184]. This suggests that these cells contribute to circulating NAMPT, although no method for NAMPT secretion has been robustly identified [212]. Chapter 5 examined the expression and release of NAMPT by neutrophils. qPCR and immunoblotting were used to measure NAMPT mRNA and protein in healthy neutrophils, both resting and stimulated with inflammatory cytokines, and also from the peripheral blood and synovial fluid neutrophils of RA patients. The aims were to establish whether neutrophils regulate NAMPT expression and release in response to cytokines, and also to examine the dynamics of NAMPT expression in the neutrophils of RA patients at different stages of disease. Expression of NAMPT transcripts was increased slightly by cytokine stimulation of neutrophils, but immunoblotting for NAMPT protein was problematic due to degradation of the protein, and no significant effects of cytokine treatment were observed. NAMPT is also constitutively expressed at high levels in resting neutrophils, by a mechanism that can be blocked by the NF-κB inhibitor, BAY-11. Transcriptome sequencing of resting, healthy neutrophils showed that only a small number of genes exhibited higher expression than NAMPT, indicating that it is one of the most abundant transcripts in neutrophils. NAMPT was, however, not readily detected in neutrophil cell culture medium, suggesting that neutrophils do not actively

secrete this protein. However, because neutrophils appear to express high basal levels of NAMPT, that may be further augmented by cytokine treatment, NAMPT could be released during cell death. Neutrophil apoptosis is not appropriately regulated during chronic inflammation, and secondary necrosis of immune cells may well contribute to the high concentration of NAMPT reported at inflammatory loci, such as in the rheumatoid joint. It was shown here, that activated synovial fluid neutrophils contain significantly more NAMPT protein than peripheral blood neutrophils from the same patient. Thus, the combination of inflammatory stimuli in the multicellular environment of the rheumatoid joint, plus the effects of transmigration, appear to increase NAMPT expression in a manner that was not observed with single cytokine stimulation *in vitro*.

NAMPT expression by peripheral blood neutrophils of RA patients did appear to correlate with expression of TNFα in patients whose disease was not well controlled by DMARD therapy. This correlation did not hold following commencement of biologic therapy (with either Rituximab or Adalimumab) and expression of neither molecule correlated with the patients disease activity score before or after treatment, in this small cohort. RA is a heterogeneous disease driven by a variety of inflammatory mediators, but as NAMPT protein appears to be elevated in RA synovial fluid neutrophils and NAMPT RNA correlates with TNFα in some RA

patient neutrophils preliminarily identified here, it could be suggested that elevated NAMPT expression indicates an activated neutrophil phenotype. As neutrophils contribute to tissue-damage in RA, this supports the hypothesis that targeting NAMPT may be beneficial to down-regulate neutrophil-mediated joint damage. This preliminary study of RA patients, however, only contains small numbers of patients and a larger patient cohort would be required to validate these conclusions.

In view of the effects of NAMPT inhibition on neutrophil function, experiments in Chapter 6 examined the global impact of NAMPT on neutrophil gene expression. NAMPT appeared to stimulate low-level increases in activation of signalling molecules NF-κB and STAT3, although this did not affect expression of the key neutrophil cytokines analysed. In contrast, inhibition of NAMPT significantly decreased activation of NF-κB, ERK1/2 and STAT3, and initial experiments showed that it also decreased expression of TNFα in response to stimuli. These observations were expanded by mRNA sequencing of healthy neutrophils to compare the gene expression profile of resting cells to those stimulated with TNFα or pre-treated with FK866 prior to TNFα stimulation. Although this experiment was only conducted once, many of the observed changes correlated with the effects on neutrophil function determined in this thesis and in the wider-literature. For example, NAMPT inhibition significantly affected the NRF2 pathway which is mediated by ROS, which was shown

to be decreased by NAMPT inhibition in Chapter 3 of this thesis. FK866 pre-treated cells exhibited a more pro-apoptotic expression profile than those treated with TNFα, and this correlates with the anti-apoptotic action of this molecule reported in the literature. NAMPT-inhibition prior to TNFα treatment down-regulated many of the TNF-induced neutrophil responses, including production of cytokines and chemokines. However, the decrease in expression was not global, suggesting a more targeted effect of this inhibitor, rather than just NAD depletion affecting the basal transcriptional machinery of the cell. This could be investigated further by targeting a different enzyme involved in NAD biosynthesis. It would also be of interest to examine the effects of NAMPT inhibition on transcription stimulated by different cytokines. These FK866-mediated effects on the neutrophil transcriptional profile further support the hypothesis that NAMPT inhibition may be beneficial in chronic inflammatory disease. The changes in gene expression with FK866 suggest a neutrophil phenotype that would produce less inflammatory mediators and be more inclined to undergo apoptosis, both of which would be expected to dampen the chronic immune response *in vivo*.

The NAMPT inhibitor FK866 was initially developed as an anti-cancer agent, and has recently completed phase II clinical trials for the treatment of solid and haematological malignancies, with a good safety profile [231,232]. There is precedent for drugs initially developed for the treatment of cancer

to be used successfully for the treatment of inflammatory diseases such as RA. The most notable example of this is methotrexate, which at low-doses can be very effective at managing RA [271,272].

In conclusion, this research has identified that NAMPT expression by neutrophils is increased in the rheumatoid joint, and correlates with TNFα in RA patient blood neutrophils, suggesting NAMPT may be a marker of inflammation in these cells. Secondly, this work has shown that NAMPT regulates neutrophil function via its role as an NAD biosynthetic enzyme. Inhibition of NAMPT decreases the amount of potentially tissue-damaging ROS produced by neutrophils, without adversely affecting the ability of these cells to kill bacteria, and it also down-regulates neutrophil expression of cytokines and chemokines. Further research is required to establish the mechanisms by which NAMPT inhibition and NAD depletion affect neutrophil functions and gene expression, but taken together these data suggest that NAMPT may be a viable target for the treatment of chronic inflammatory diseases such as RA, and that FK866 may represent a promising new therapy.

A number of other inhibitors of the NAMPT enzyme have emerged over recent years, including GMX1778 (a pyridyl cyanoguanidine compound) and CB-30865 (a quinazoline-based pyridine-containing compound) [273], and a high-throughput screening mechanism has been developed to discover novel NAMPT inhibitors [274] . So going forward from the work

carried out in this thesis it would be of interest to verify the findings of this work using alternative inhibitors of NAMPT enzyme function. However, before NAMPT inhibitors can be considered as a viable option for treating inflammatory diseases, such as RA, the global implications of blocking this pleiotropic enzyme need to be further explored, both *in vitro* and ultimately *in vivo*.

7.1 Future directions

One way in which knowledge could be expanded on the global effects of NAMPT on neutrophils would be to silence the expression of this enzyme using RNA interference. Utilising small interfering RNA (siRNA) would also remove potential confounding, off-target effects of the NAMPT inhibitor. NAMPT gene knock-out in mice is embryonic lethal [26,31,174], however targeted knock-down of the NAMPT gene in neutrophils or neutrophil-like cell lines, would allow a comprehensive study into the role of NAMPT in these cells. Previous work has demonstrated that transfection of *ex-vivo* neutrophils can be unreliable and problematic due to the tendency of these cells to engulf particles by phagocytosis, so the use of a neutrophil-like cell line may be more practical for RNA interference studies. The human promyelocytic HL-60 cell line can be induced to differentiate into neutrophil-like cells in response to a variety of chemical stimuli such as retinoic acid and dimethylsulphoxide [275].

To expand this work further, it would also be of interest to study the effects of either NAMPT inhibited or NAMPT depleted neutrophils in a multicellular model, more representative of the rheumatoid joint *in vivo*. This would provide some insight into the role of NAMPT produced by neutrophils in paracrine signalling. To produce a more representative model of the rheumatoid joint *in vitro* incubations of NAMPT inhibited or depleted neutrophils could be carried out in rheumatoid synovial fluid and under hypoxic conditions. Previous work has identified that *in vitro* neutrophil transmigration towards IL-8 prior to incubation in inflammatory synovial fluid is preferable to incubation with freshly isolated cells (C. Lam and H. Wright, unpublished data).

NAMPT inhibition has also been reported to exert effects on a number of immune cell types critical to the establishment and progression of RA [18,19,22], and some cells such as T-lymphocytes have been described as exquisitely sensitive to NAMPT inhibition [223]. So, to truly understand the role of NAMPT in chronic inflammation and the potential of NAMPT inhibition to alleviate the chronic inflammatory state, it would be of interest to explore similar studies to those conducted in this thesis, using other inflammatory cell types in isolation or as part of a multicellular model.

APPENDIX

Gene Symbol	Entrez Gene Name	RPKM expression values			Fold Change TNF to T+FK	Type(s)
		Control	TNF	T+FK		
IL1A	interleukin 1, alpha	0.54	47.40	1.74	-27.32	cytokine
GPR84	G protein-coupled receptor 84	1.19	120.65	6.85	-17.62	G-protein coupled receptor
IL1B	interleukin 1, beta	333.14	3462.04	203.50	-17.01	cytokine
IL1RN	interleukin 1 receptor antagonist	90.59	1162.98	77.52	-15.00	cytokine
FOSL1	FOS-like antigen 1	7.47	14.38	0.89	-14.38	transcription regulator
ARL5B	ADP-ribosylation factor-like 5B	45.19	114.33	9.08	-12.59	enzyme
PTGS2	prostaglandin-endoperoxide synthase 2	252.81	669.66	53.87	-12.43	enzyme
KCNJ2	potassium inwardly-rectifying channel, J2	11.33	152.93	17.39	-8.80	ion channel
EREG	epiregulin	4.04	19.92	2.39	-8.35	growth factor
XBP1	X-box binding protein 1	6.91	47.27	5.67	-8.34	transcription regulator

TABLE 6.1: Protein coding transcripts showing greatest decrease in expression (from control) when neutrophils are pre-treated with FK866 prior to TNFα stimulation

Gene Symbol	Entrez Gene Name	RPKM expression values			Fold Change TNF to T+FK	Type(s)
		Control	TNF	T+FK		
GADD45G	growth arrest and DNA-damage-inducible, gamma	6.57	1.98	19.05	9.61	other
HHEX	hematopoietically expressed homeobox	10.93	3.42	30.49	8.91	transcription regulator
STX6	syntaxin 6	14.43	9.20	81.75	8.89	transporter
BMP6	bone morphogenetic protein 6	3.40	2.99	24.91	8.32	growth factor
HMOX1	heme oxygenase (decycling) 1	12.40	6.96	49.42	7.10	enzyme
ENC1	ectodermal-neural cortex 1 (with BTB-like domain)	34.81	10.54	65.31	6.20	peptidase
RPL21	ribosomal protein L21	5.72	0.00	6.16	6.16	other
PROC	protein C (inactivator of coagulation factors Va and VIIIa)	1.03	0.40	6.15	6.15	peptidase
ZHX2	zinc fingers and homeoboxes 2	6.00	5.65	33.82	5.98	transcription regulator
FHL3	four and a half LIM domains 3	22.12	15.82	93.55	5.91	other

TABLE 6.2: Protein coding transcripts showing greatest increase in expression (from control) when neutrophils are pre-treated with FK866 prior to TNFα stimulation

Gene Symbol	Entrez Gene Name	RPKM expression values			Fold change from control		Fold change from TNF
		Control	TNF	T+FK	TNF	T+FK	T+FK
ACTB	Actin, beta	2721.26	2556.07	2152.97	-1.06	-1.26	-1.19
B2M	Microglobulin, beta 2	12414.40	11816.7	11744.9	-1.05	-1.06	-1.01
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	265.22	215.59	379.20	-1.23	1.43	1.76
HPRT1	Hypoxanthine phosphoribosyltransferase 1	2.61	2.50	1.59	-1.04	-1.64	-1.57
RPLP0	Ribosomal protein, large, P0	35.18	33.71	26.55	-1.04	-1.32	-1.27

TABLE 6.5: Expression of a selection of housekeeping genes across all treatments

Upstream TF	Fold Change	p-value	Target molecules in dataset
NFkB complex		1.56E-32	BBC3,BCL2A1,BCL3,BID,BIRC3,CCL20,CCL3,CCL3L1/CCL3L3,CCL4,CD274
RELA	1.635	2.40E-26	BBC3,BCL2A1,BCL3,BIRC3,BTG2,CCL20,CCL3,CDKN1A,CDKN1B,CXCL1
NFKB1	1.376	2.95E-22	BCL2A1,BCL3,BIRC3,BTG2,CCL20,CCL3L1/CCL3L3,CCL4,CDKN1A,CXCL2,CXCL3
REL	1.933	4.38E-17	BBC3,BCL2A1,BCL3,CCL4,CDKN1A,F3,GADD45B,ICAM1,IER3,IL18
JUN	-1.055	6.95E-16	ACAT2,ACP5,BBC3,BCL2A1,BCL3,BIRC3,BTG1,C3AR1,CD14,CD274
EGR1	-1.224	4.39E-13	CCL3L1/CCL3L3,CCL4,CDK5R1,CDKN1A,CXCL2,CXCL3,ELK1,F3,FOSL1,GADD45B
HMGB1	-1.189	1.37E-11	CCL20,CCL3,CCL4,CD83,CDKN1A,CXCL3,ICAM1,IL1A,IL1B,IL8
FOXL2		4.33E-11	BCL2A1,CCL20,CCL3,CCL3L1/CCL3L3,CXCL2,CXCL3,ICAM1,IER3,MAFF,PPP1R15A
STAT3	-1.071	1.58E-10	BCL3,CCL20,CCL3L1/CCL3L3,CCL4,CD274,CD83,CDKN1A,CDKN1B,CXCL2,FFAR2
CEBPB	1.186	2.59E-09	BCL2A1,CCL3,CCL3L1/CCL3L3,CCL4,CD14,CDC42EP3,CDKN1A,CDKN1B,CLEC4E,CXCL2
CEBPA	-1.480	1.52E-08	BCL2A1,BTG1,BTG2,C3AR1,CCL20,CD14,CDKN1A,CKAP4,G0S2,GBP1
CREB1	-1.006	2.19E-08	BTG2,CD79A,CSRNP1,CXCL2,DUSP1,GADD45B,HMOX1,IL1B,JUNB,LDLR
Ap1		2.57E-08	BCL2A1,C3AR1,CCL3,CCL3L1/CCL3L3,CCL4,F3,FOSL1,HMOX1,IL1A,IL1B
NfkB-RelA		3.54E-08	CCL3,CXCL3,ICAM1,IL1B,IL8,NFKBIA,PTGS2,TNF
NFKB2	3.095	4.68E-06	BIRC3,CCL20,CDKN1A,IL8,PTGS2,SOD2,TNF,TRAF1

TABLE 6.6: Transcription factors identified by IPA as activated with TNFα treatment

Upstream TF	Fold Change	p-value	Target molecules in dataset
NFkB complex		5.92E-13	ABCG1,ATF3,B3GNT5,BAX,BBC3,BCL11A,BCL2A1,BCL2L11,BCL3,BIRC3
RELA	-1.412	5.19E-09	ACTN4,BBC3,BCL2A1,BCL3,BIRC3,BTG2,CARD8,CASP8,CCL20,CCL3
HMGB1	-1.368	4.73E-08	BAX,BRCA1,CCL20,CCL3,CCL4,CCNG1,CD58,CD83,CXCL3,ICAM1
CREB1	-1.107	4.91E-08	ABCA1,ATF3,ATP1A1,BHLHE40,BTG2,CCND3,CD68,CEBPB,CEBPD,CIITA
REL	-2.721	7.41E-08	AHR,BBC3,BCL2A1,BCL2L11,BCL3,CCL4,CIITA,E2F3,EGR2,F3
NFKB1	1.978	3.05E-06	BCL2A1,BCL3,BIRC3,BTG2,CCL20,CCL3L1/CCL3L3,CCL4,CCL5,CIITA,CREB3
FOXL2		7.96E-05	ATF3,BCL2A1,CCL20,CCL3,CCL3L1/CCL3L3,CXCL2,CXCL3,FOS,HRH2,ICAM1
NfkB-RelA		4.48E-04	CCL3,CCL5,CXCL3,ICAM1,IL1B,IL8,NFKBIA,PTGS2,TNF
SREBF1	1.653	3.66E-03	ABCA1,ACLY,BAX,CEBPA,CEBPB,CEBPD,DARS,DHCR7,FAS,FDFT1
BCL10	-1.476	5.25E-03	CCL4,CCL5,CXCL2,IL8,TNF
JUND	-2.053	2.81E-02	BCL3,CCL5,CYBB,F3,FOSL1,HMOX1,JUND,NCF2,NR4A1,PLAUR
Ap1		4.05E-02	ATF3,BCL2A1,BCL2L11,CCL3,CCL3L1/CCL3L3,CCL4,F3,FOS,FOSL1,GSTP1
NfkB1-RelA		4.27E-02	CCL5,CXCL1,ICAM1,IL8,PTGS2,TNF
FOSL2	1.201	4.33E-02	ABCA1,CEBPB,F3,FAS,FOSL1,GCM1,IL8,RELB
TRIM24	-1.691	9.18E-02	CMPK2,DHX58,EIF5A,HERC6,IFIH1,IFIT2,IFIT3,IRF7,IRF9,JAK2

TABLE 6.7: Transcription factors identified by IPA as inhibited with FK866 prior to TNFα treatment

Upstream TF	Fold Change	p-value	Target molecules in dataset
GFI1	1.000	3.20E-11	BCL3,CDKN1A,CDKN1B,CEBPE,ETS2,ICAM1,IL1A,IL1B,IL8,IRAK2
MEOX2		4.03E-11	CCL20,CD69,CDKN1A,CXCL1,CXCL2,CXCL3,F3,ICAM1,IL8,PTX3
ZFP36	4.626	1.01E-06	BIRC3,CCL3L1/CCL3L3,CDKN1A,IL1B,PTGS2,TNF
KLF2	-1.048	5.10E-06	BCL3,CCL3,CCL4,CDKN1A,F3,HYAL2,IL1B,IL8,JUNB,NFKBIA
NRF1	-1.004	4.32E-03	CCL20,HMOX1,IL1A,IL1RN,IL8
Nr1h		8.95E-03	CCL3,CXCL1,IL8,PTGS2,TNF

TABLE 6.8: Transcription factors identified by IPA as inhibited with TNFα treatment

Upstream TF	Fold Change	p-value	Target molecules in dataset
IRF3	-1.516	4.88E-05	AHNAK,B4GALT5,BIRC3,CCL3,CCL3L1/CCL3L3,CCL4,CCL5,CD58,CLIC4,CXCL1
MEOX2		1.14E-04	CCL20,CD69,CDKN2D,CXCL1,CXCL2,CXCL3,CXCL6,F3,ICAM1,IL8
FOSL1	-14.375	2.22E-04	CCL3L1/CCL3L3,CCL4,CXCL6,EGR1,EGR2,FOS,FOSB,FOSL1,GCLC,GCM1
TFEB	2.167	3.26E-03	ATP6V1H,CLCN7,CTSD,GBA,HEXA,MCOLN1,SGSH,TPP1,VEGFA
ZFP36	-2.819	4.75E-03	BIRC3,CCL3L1/CCL3L3,FOS,IL1B,PTGS2,TNF
HDAC5	1.058	5.10E-03	CASP8,CDK7,HES1,HK2,MAP3K3,MAPK7,MEF2C,PTEN,TNF,TNFRSF1A
Nr1h		6.51E-02	ABCA1,ABCG1,ACSL3,CCL3,CCL5,CXCL1,IL8,PTGS2,SREBF1,TNF
BCL3	-2.405	1.97E-01	CCL5,IL1B,IL8,PLAUR,TNF
LHX1	1.000	1.00E00	ANPEP,JAG1,KCNJ15,ZKSCAN1

TABLE 6.9: Transcription factors identified by IPA as activated with NAMPT inhibition prior to TNFα treatment

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