THE ROLE OF INHIBITORY KAPPA B KINASE ALPHA (IKKα) IN THE REGULATION OF NUCLEAR FACTOR KAPPA B SIGNALLING IN ENDOTHELIAL CELLS

By

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ABSTRACT

Atherosclerosis is one of the most common cardiovascular diseases in Western Countries. An important feature of the disease is the initiation of atherosclerotic lesions including monocyte recruitment into the vessel wall (Cominacini et al., 2000). This involves the expression a number of adhesion molecules including E-selectin, ICAM-1 and VCAM-1. In endothelial cells, ICAM-1 and VCAM-1 expression is regulated by a number of defined signalling pathways including the transcription factor Nuclear factor Kappa B (NFκB). NFκB activation is regulated by α and β isoforms of the Inhibitory Kappa B kinases (IKK). Whilst IKKβ regulates the canonical pathway involving phosphorylation and degradation of IκB-α and the translocation of p65 NFκB to the nucleus, accumulated evidence implicates that IKKα regulates p65 dependent gene expressions and plays a distinctive role in non-canonical NFκB pathway by mediating processing of p100 NFκB precursor p100. In this PhD thesis, I have examined the effect of adenoviral (Adv.) mediated IKKα and β blockade or siRNA IKKα on NFκB activation and adhesion molecule expression in endothelial cells.

In human umbilical vein endothelial cell, over-expression of Adv. DN-IKKβ attenuated TNF-α induced IκB-α degradation and p65 phosphorylation. In contrast, Adv. DN-IKKα had no inhibitory effect of either responses. Furthermore, either Adv. DN-IKKα or siRNA IKKα reduced TNF-α induced NFκB reporter activity and also p65-dependent expression of the adhesion molecules, ICAM-1 and VCAM-1. Taken together, these findings suggest IKKα may regulate NFκB activity and gene expression without being involved in the up-stream canonical NFκB pathway. Moreover, blocking IKKα but not β inhibited TNF-α induced activation of the non-canonical pathway exemplified by p100 processing and the formation of p52. Novel selective IKKα inhibitors, SU1007 and SU1010, generated in house, were employed in the study to confirm the role of IKKα in endothelial cells. Similarly, both SU compounds did not affect TNF-α induced IκB-α degradation and phosphorylation of p65 but inhibited NFκB reporter activity and ICAM-1 and VCAM-1 expression. In addition, SU 1007 and 1010 also showed an inhibitory effect on the non-canonical pathway by blocking both p100 processing and the expression of p100 itself.

These findings indicate IKKα plays a key role in the regulation of the non-canonical NFκB pathway and regulates the canonical NFκB pathway via its nuclear activity. These data

suggest that IKKα inhibition may be a useful approach for the future treatment of cardiovascular disease.

PUBLICATIONS

Original Research Articles

Ka Ho Ho, Rebecca Scott, Sophia Hatziieremia, Kathryn McIntosh, Louise Young, Alan Harvey, Judith Huggan, Nahoum Anthony, Stuart Lang, David Breen, Murray Robertson, Oliver Sutcliffe, Simon Mackay, Colin Suckling, Joanne Edwards, Andrew Paul and Robin Plevin* (2012). Novel compounds with selectivity for inhibitory Kappa B kinase 1 regulate inflammatory gene expression in human endothelial cells. (In Preparation)

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ABBREVIATIONS

Adv.	Adenovirus
ANOVA	Analysis of variance
AKT	Serine/threonine-protein kinase
APS	Ammonium persulphate
Ang-II	Angiotensin-II
ATF-2	Activating transcription factor
BAFF	B cell activation factor
BAD	Bcl-2 assoicated death promoter
BLC	B lymphocyte chemoattractant
BSA	Bovine serum albumin
Bcl-2	B-cell lymphoma-2
BMK1	Big MAPK-1
CBP	CRE binding protein
CCL	C-C motif ligands
CHIP	Chromatin Immunoprecipitation
CXCL-12	Chemokine (C-X-C motif) ligand
cGMP	Cyclin guanosine monophosphate
CDK4	Cyclin-dependent kinase 4
COX	Cyclooxygenase
DN	Dominant negative
DTT	Dithiothreitol
eNOS	Endothelial nitric oxide synthase

ECL	Enhanced Chemiluminescence
ELC	EBI-1-ligand chemokine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
Elk-1	ETS-like transcription factor 1
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GPCRs	G-protein coupled receptors
GSK3	Glycogen synthase kinase-3
H_2O_2	Hydrogen peroxide
HEK	Human embryonic kidney
HLH	Helix-loop-helix
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intracellular adhesion molecule-1
IL-1β	Interleukin-1 beta
IL-1R	Interleukin-1 receptor
IκB-α	Inhibitory kappa B alpha
IKK	Inhibitory kappa B kinase
JNK	c-Jun N-terminal kinase
kDa	kilo-Dalton
KDIF	Keratinocyte differentiation-inducing factor

LDL	Low density lipoprotein
Luc	Luciferase
LTα1β2	Lymphotoxin alpha 1 beta 2
MMP	Matrix metalloproteinases
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MEK	MAP (Mitogen-Activated Protein) Kinase/ERK (Extracellular Signal-Regulated Kinase) Kinase
MEF	Mouse Embryonic Fibroblast
NFκB	Nuclear factor kappa B
NEMO	NFκB essential modulator
NIK	NFκB inducing kinase
NLS	Nuclear localisation sequence
NO	Nitric oxide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDTC	Pyrrolidine dithiocarbamate
PMA	Phorbol 12-myristate 13-acetate
PP2A	Protein phosphatase 2A
PTEN	Protein Tyrosine Phosphatase
PI3K	Phosphoinositide-3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate

PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PGI_2	Prostacyclin
PKC	Protein kinase C
SCC	Squamous cell carcinomas
SLC	Secondary lymphoid tissue chemokine
SDS	Sodium dodecyl sulphate
SP-1	Specific protein-1
siRNA	Small interfering RNA
shRNA	Short hairpin interfering RNA
RANK	Receptor activator of NFκB
TAB-2	TAK-1 binding protein 2
TAK-1	Transforming growth factor β–activated kinase 1
TEMED	N,N,N',N'-tetramethylenediamine
TGFR	Transforming growth factor β recepotr
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
TNFR	TNF-receptor
TRAF	TNF receptor-associated factor
UBF	Upstream binding factor
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

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Chapter 1

General Introduction

1.1 Physiology of the Endothelium

In multicellular organisms, blood vessels are essential for the function of the circulatory system. Oxygen and nutrients are delivered from blood vessels to various tissues, while waste products are removed from tissues to the circulation and then excreted from the body. The circulation system is also responsible for inter-organ communication such as transporting hormones from one organ to another. Vascular endothelium is a thin layer of endothelial cells that lines the tunica intima of the inner layer of the blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells line the entire circulatory system, from the heart to the smallest capillary. The endothelial cell surface in an adult human is composed of approximately 1 to 6 x 10^{13} cells, weighs approximately 1kg, and covers a surface area of approximately 1 to 7 m^2 (Augustin et al., 1994, Fishman, 1982). One of the roles of vascular endothelium is in delivering oxygen and nutrients to various underlying tissues and removing wastes from tissues. There is also a reciprocal relationship between the endothelium and its surrounding microenvironment which is important not only during organ development and patterning, but also in regulating the local immune response under pathological conditions (Baeuerle and Baltimore, 1988, Nikitenko, 2009).

The endothelium plays a key role in the regulation of blood pressure. Its presence reduces turbulent blood flow by controlling blood vessel contraction and relaxation (Stankevicius et al., 2003). Both vasoconstriction and vasodilatation are crucial in the regulation of blood pressure. Vasoconstriction is regulated by vasoactive molecules such as angiotensin II and vasopressin which act on their cognate receptors to induce vascular smooth muscle contraction, thus reducing the size of lumen and hence raising blood pressure. However the propensity towards vasoconstriction must be counterbalanced by vasodilatation, otherwise blood vessels would be damaged under high blood pressure over a prolonged period. Vasodilatation is mediated by the synthesis and release of key endothelial derived relaxing factors such as nitric oxide (NO) (Harrison, 1997, Kubes et al., 1991, Kugiyama et al., 1996), prostacyclin (PGI_2) (Camacho et al., 2008, Jaffe and Weksler, 1979) and epoxyeicosatrienoic acids (Stankevicius et al., 2003) which migrate into the vascular smooth muscle layer and cause relaxation of the vascular smooth muscle. For example, NO diffuses into the smooth muscle cell and activates soluble guanylyl cyclase to increase intracellular cyclic guanosine monophosphate (cGMP) concentration. An increase of cGMP level results in a decrease of

the intracellular calcium concentration in smooth muscle cells leading to relaxation (Stankevicius et al., 2003). In contrast, prostacyclin (PGI_2), released by the endothelial cell through arachidonic acid metabolism, regulated in turn by phospholipase A_2 and cyclooxygenase (COX) activity, interacts with specific receptors on the smooth muscle surface resulting in increases in intracellular cAMP and subsequent relaxation (Coleman et al., 1994). The effects of both NO and PGI_2 are strongly related to each other, PGI_2 facilitates NO release from endothelial cells, while NO potentiates PGI_2 effects in smooth muscle (Stankevicius et al., 2003).

1.2 Dysfunction of the Endothelium

A number of risk factors for cardiovascular disease contribute to endothelial dysfunction such as smoking, hypertension, hypercholesterolaemia, diabetes and obesity. Moreover, physical inactivity can also lead to endothelial dysfunction (Suvorava et al., 2004). This is linked to cellular changes for example, disregulation of NO synthesis is associated with these conditions and also diabetes, shock, infarction, neurodegeneration, arthritis and chronic inflammation (Gross and Wolin, 1995). Endothelial dysfunction also leads to decreased secretion of vasodilator mediators, and increased production of endothelium-derived vasoconstrictors, and/or resistance of vascular smooth muscle to vasodilators (Mombouli and Vanhoutte, 1999). In addition to promoting vasodilation, normal endothelium has anti-atherosclerotic and anti-thrombotic functions. It inhibits platelet aggregation, monocyte adhesion, vascular smooth muscle cell proliferation, and thrombosis, all of which are important factors in the development of atherosclerosis and eventual plaque disruption (Coleman et al., 1994, Flavahan, 1992, Levine et al., 1995, McGorisk and Treasure, 1996, Vane et al., 1990, Vogel, 1997). Endothelial dysfunction and atherosclerosis will be discussed further in section 1.6.5.

The initial stage of atherosclerosis is believed to be associated with the dysfunction or activation of vascular endothelial cells (Dzau et al., 2002, Libby et al., 2010b, Ross, 1999). In the latter stage, endothelial cells are associated with the deposition of lipids such as low density lipoprotein (LDL) and cholesterol, infiltration of inflammatory cells, and proliferation of vascular smooth muscle cells (Dzau et al., 2002, Libby et al., 2010b, Ross, 1999). Unstable atherosclerotic plaques become susceptible to rupture that contributes to the formation of thrombus and this may lead to more severe events such as myocardial infarction (Dzau et al.,

2002, Libby et al., 2010b, Ross, 1999) and stroke (Fadini et al., 2007). Infiltrating macrophages and T lymphocytes produce inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) which regulate endothelial cell responses. This includes the induction of cellular adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) on the endothelial cell surface, which in turn mediates recruitment of circulating leukocytes to the arterial wall (Libby et al., 2010b, Price and Loscalzo, 1999, Ross, 1999). Endothelial cells along with macrophages and vascular smooth muscle cells release matrix metalloproteinases (MMPs) which increase the degradation of the components of the fibrous cap covering the atherosclerotic plaque and contributes to plaque disruption (Libby, 2002, Saren et al., 1996).

The role of the endothelium is not restricted to cardiovascular disease. There is evidence in the cancer field that endothelial cells provide support for tumour growth and metastatic spread. Excessive proliferation and transformation of endothelial cells can lead to pathological angiogenesis or lymphangiogenesis or vascular malfunctions, which are now considered to be the hallmarks of malignant disorders (Nikitenko, 2009).

1.3 Signalling mechanisms in Endothelium

There are several intracellular signalling events that are required for endothelial cells to induce different physiological responses. These responses are initiated by a considerable number of extracellular agents including; growth factors such as vascular endothelial growth factor (VEGF), Fibroblast growth factor (FGF) and Epidermal growth factor (EGF), cytokines including TNF-α and IL-1β, activators of G-protein coupled receptors (GPCR) such as endothelin-1 (ET-1), angiotensin-II (Ang-II) and vasopressin and stimuli such as hypoxia, oxygen free radicals and cellular distension. Cellular activation by these agents converge on a number of key intracellular signalling pathways which are essential in the regulation of endothelial cell function. In particular, the Mitogen Activated Protein Kinase (MAPK), Phosphoinositide-3 Kinase (PI3K) and Nuclear Factor Kappa B (NFκB) pathways have been strongly implicated. These pathways are intimately linked to cell inflammation, proliferation, migration and apoptosis and as they are tightly regulated in normal conditions, over-activation of these pathways leads to development of many diseases such as arthritis and cancers. Therefore studying the signalling mechanisms involved in endothelial cell function provides valuable information for targeting and treatment of diseases.

1.3.1 Mitogen-Activated Protein Kinase (MAPK) Signalling Pathways

The MAPKs superfamily is a major system participating in the transduction of signals from the cell membrane to nuclear and other intracellular targets. MAPKs respond to numerous stimuli by phosphorylating a variety of substrates including transcription factors, enzymes, and other kinases. They are a three-tiered kinase cascade comprising a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and an effector MAPK. The most well-known MAPK signalling pathways included the extracellular regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and the p38 MAP kinase pathway (Qi and Elion, 2005). There are numerous isoforms of each kinase in particular, JNK and p38, and multiple MAPKKKs and MAPKKs which allow for specific and overlapping regulation of each MAPK (Qi and Elion, 2005). Figure 1.1 outlines each of the MAPK signalling pathways. The members of the MAPK families are responsible for regulation of a large variety of cellular processes such as cell growth, differentiation, development, cell cycle regulation, death and survival. While ERK1/2 and also Big MAPK-1 (BMK1) play an important role in growth and cytoprotective functions, JNK and p38 proteins are implicated in cell death, inflammatory and stress responses. Because they have predominantly contradictory roles, the relative activation of these kinases is important to the functional status of the cell. Additionally, there is known to be a crosstalk between MAPK cascades whereby the activity of one MAPK can be influenced by another. Thus, these proteins collectively integrate the pro- and anti-inflammatory stimuli acting on the cell to produce the appropriate downstream effects (Hoefen and Berk, 2002).

Whilst not examined directly in this thesis, there is evidence to show that MAP kinases play a role in endothelial cell function. Pro-inflammatory cytokines or genotoxic stress such as ionising radiation or cisplatin can induce JNK translocation into the mitochondria from the cytoplasm. JNK then phosphorylates numerous targets such as Bcl-X, Bax and 14-3-3σ (Cadalbert et al., 2005, Dhanasekaran and Reddy, 2008, Kharbanda et al., 2000, Kim et al., 2006, Yamamoto et al., 1999). These proteins are involved in release of cytochrome-c which is a crucial step for the initiation of apoptosis. Some studies showed inhibition of JNK activity leads to a reduction of apoptosis in cardiomyocytes. (Remondino et al., 2003, Aoki et al., 2002). Hydrogen peroxide (H_2O_2) activates SAPK/Erk kinase (SEK1)/JNK phosphorylation and hence activates caspase-3 and leads to apoptosis. Inhibiting JNK activity by dominant negative JNK (DN-JNK) and SEK1 adenovirus however showed reverse of

apoptosis (Murakami et al., 2005). Although JNKs plays an important role in the regulation of apoptosis, JNKs are also involved in the regulation of pro-survival activity. Inhibition of JNK1/2 using a synthetic peptide (JNKI 1) specifically inhibits JNK1 phosphorylation and leads to an increase in HUVECs apoptosis, although the same inhibitor did not affect cell migration or proliferation (Salameh et al., 2005). Therefore, JNK has an essential role in balancing both the pro- and anti-apoptotic pathways.

Another important MAPK sub-group is ERK. There are six isoforms of ERK but the most studied among these are ERK-1, ERK-2 and ERK-5. ERK signalling can be activated by a wide range of stimuli through activation of a number of different receptors such as epidermal growth factor receptor (EGFR), GPCRs and transforming growth factor β receptor (TGFR) (Schramek, 2002). ERK1 and ERK2 are activated by MAP (Mitogen-Activated Protein) Kinase/ERK (Extracellular Signal-Regulated Kinase) Kinase 1 (MEK1) and MEK2 (Zheng and Guan, 1993) and regulate several transcription factors such as E-twenty six (ETS)-like transcription factor 1 (Elk-1) (Marais et al., 1993), c-myc (Gupta et al., 1993) and upstream binding factor (UBF) (Stefanovsky et al., 2001) which are important in the regulation of cell survival, proliferation and differentiation. In fact, ERKs play an important role in endothelial cell proliferation and migration. Endothelial cells from ERK1 and 2 double knockout mutant embryos showed a 3-fold decrease in proliferation of endothelial cells compared with wild type (Srinivasan et al., 2009). Moreover, a marked diminution of proteins regulating G1 to S phase progression was observed, including cyclin D1, cyclin E, Cyclin-dependent kinase 4 (CDK4) and c-Myc (Srinivasan et al., 2009). In addition, an *in vitro* experiment showed endothelial cells lacking both ERK1 and 2 had attenuated cell migration and invasion compared with control (Srinivasan et al., 2009). Furthermore, the MEK inhibitor PD184352, inhibited phosphorylation of ERKs and induced HUVECs apoptosis. Over-expression of dominant-negative MEK1 (MEK1A) in HUVECs also inhibited phosphorylation of ERKs and lead to failure of tubule formation (Mavria et al., 2006). Overall, these studies outlined above confirm the role of ERKs in the regulation of the proliferation and migration of endothelial cells.

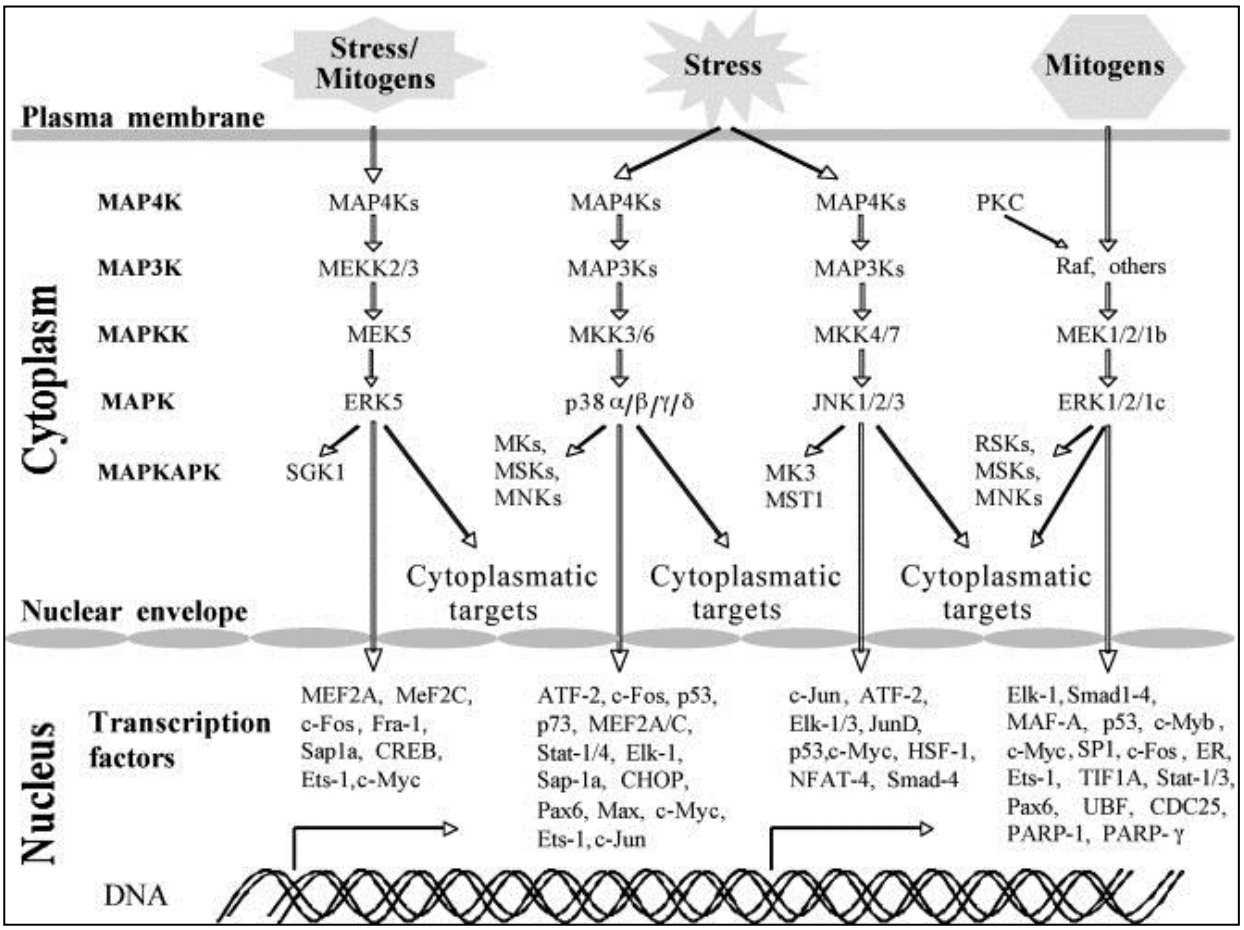


Figure 1.1 Schematic representation of the MAPK cascades and their nuclear targets. Activation of ERK1/2 signalling pathway results in activation of a series of transcription factors such as E-twenty six like transcription factor (Elk-1), specific protein 1(SP-1) and c-Myc which play an important role in the regulation of cell survival and proliferation. Whilst activation of JNKs pathway results in activation of transcription factors including c-Jun, activating transcription factor (ATF-2) for cell survival, proliferation and also apoptosis (Adapted from (Plotnikov et al., 2011)

1.3.2 The Phosphoinositide-3 Kinase Signaling Pathway

The PI3K/PTEN/AKT pathway is important in the regulation of cellular function, in particular cytoskeletal organisation and cellular survival (Gautreau et al., 1999). Cells exposed to growth factors show increased recruitment and phosphorylation of phosphoinositide-3 kinase (PI3K) by the activated receptor. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to form phosphatidylinositol-3,4,5-trisphosphate (PIP_3) which subsequently activates a serine/threonine-protein kinase (AKT) through the intermediate phosphoinositide-dependent kinase-1 (PDK1). AKT is also called protein kinase B (PKB) which phosphorylates and regulates the activity of a number of downstream targets including kinases, transcription factors and other regulatory molecules (Fayard et al., 2010). The activity of the PI3K pathway is negatively regulated by Protein Tyrosine Phosphatase (PTEN) which is implicated in a number of cancers. PTEN suppresses tumour cell growth by antagonising the action of receptor tyrosine kinases and regulates tumour cell invasion and metastasis in human brain, breast and prostate (Li et al., 1997), pancreatic ductal adenocarcinoma (Ying et al., 2011) and colorectal cancer (Byun et al., 2011). Figure 1.2 illustrates the PI3K/PTEN/AKT signalling pathway. In fact, recent evidence indicates there is cross-talk between PI3K and other signalling pathways. For instance, a number of studies show one of the targets of AKT is the Inhibitory kappa B kinase (IKK) complex which is a critical kinase in regulation of NFκB pathway (see below) (Ozes et al., 1999, Rajavashisth et al., 1995).

It is well recognised the PI3K plays a key role in endothelial cell function, in particular in response to VEGF and other growth factors. Once AKT is phosphorylated, it can down-regulate the expression of apoptotic factors such as caspase-9 (Cardone et al., 1998) and BAD (Datta et al., 1997). Furthermore, it also activates survival factors such as NFκB (Ozes et al., 1999) and apoptosis regulator bcl-2. Moreover, constitutive up-regulation of B-cell lymphoma-2 (bcl-2) expression prevents endothelial cell apoptosis in a model of capillary network formation (Pollman et al., 1999), therefore AKT is crucial for promoting in endothelial cell survival and angiogenesis. In addition, AKT is associated strongly with several cytoskeletal-mediated processes, such as regulation of actin reorganisation during VEGF-induced migration of endothelial cells and signalling by cytoskeleton-plasma membrane linker protein (Morales-Ruiz et al., 2000). AKT interacts with Ezrin which also functions as plasma membrane-actin cytoskeleton linker and aids cell survival (Gautreau et

al., 1999). In addition, PI3K/AKT signalling pathway regulates endothelial nitric oxide synthase (eNOS) activation. Dimmeler and co-workers showed inhibition of the PI3K/AKT pathway or mutation of the AKT site on eNOS protein (at serine 1177) attenuated the serine phosphorylation and prevents the activation of eNOS (Dimmeler et al., 1999). eNOS produces NO which plays an important role in the local regulation of vascular homeostasis and platelet aggregation (Moncada and Higgs, 1993). A decrease in the bioavailability of NO is a characteristic feature in patients with coronary artery disease (Zeiher, 1996) and aggravates the development of atherosclerotic lesions (Moroi et al., 1998).

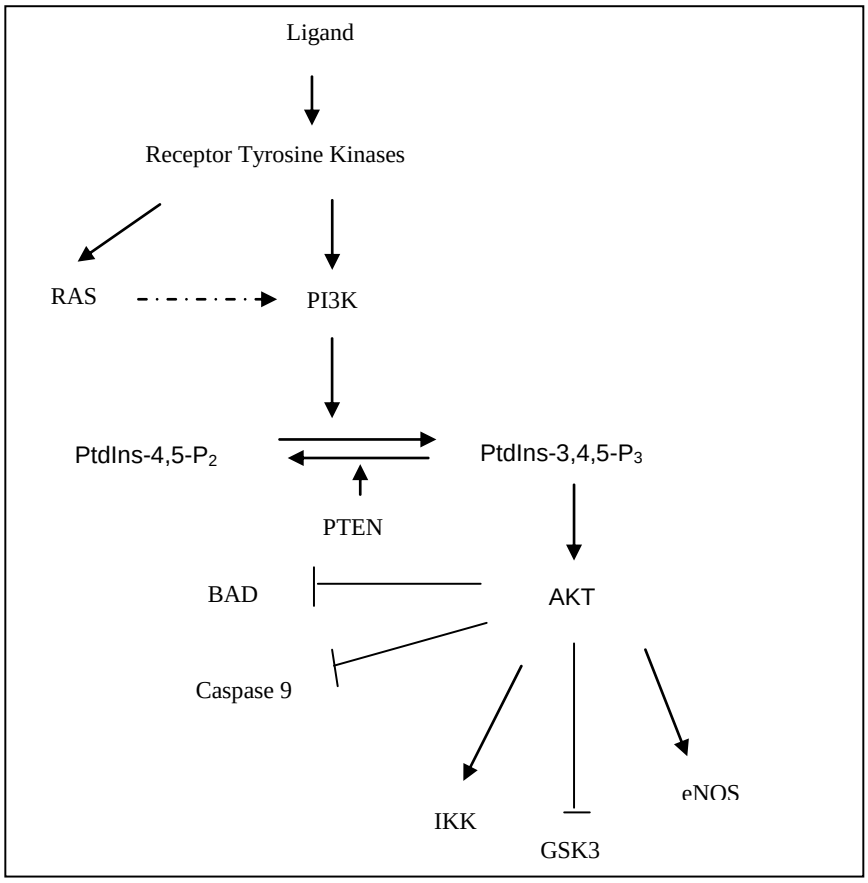


Figure 1.2 illustrates the PI3K/PTEN/AKT signaling pathway. The diagram shows the AKT is a key factor in controlling the activity of regulatory molecules. AKT regulates protective mechanism by activating the endothelial nitric oxide synthase (eNOS) and IKK in endothelial cell against vascular injury. In addition, AKT inhibits the pro-apoptotic factors including Bcl-2-assoicated death promoter (BAD) protein, glycogen synthase kinase-3 (GSK3) and caspase-9 to inhibit apoptosis.

1.3.3 The Nuclear Factor Kappa B Signalling Pathway

Nuclear factor kappa B (NFκB) is a ubiquitous signalling pathway which plays a key role in a number of both physiological and pathophysiological processes. This includes responses to infection, fetal development, cancer, inflammation, atherosclerosis and angiogenesis (Brand et al., 1996, Brand et al., 1997b). NFκB was first identified by Sen and Baltimore in 1986 (Sen and Baltimore, 1986) as a transcription factor which binds to a specific decameric DNA-sequence (5'-GGGACTTTCC-3') within the intronic enhancer of the immunoglobulin kappa light chain gene in mature B cells (Sen and Baltimore, 1986). NFκB has been found in many cell types and regulates transcriptional activity through binding to specific binding sites, termed κB sites, within the promoters of a number of genes, which have the consensus sequence 5'-GGGRNYYYCC-3', where R is purine and Y is pyrimidine (Kunsch et al., 1992, Parry and Mackman, 1994). Figure 1.3 illustrates the structure of different NFκB members.

1.3.3.1 Structure of NFκB

Early studies showed NFκB to exist as a heterodimer, purification of NFκB revealed two proteins involved in DNA binding. The molecular weight of those proteins were approximately 65kDa and 50kDa, latterly named as Rel A and p50 respectively (Ghosh et al., 1990, Kawakami et al., 1988, Zabel et al., 1991). Rel A was also called p65, while p50 named as NFκB1. Later studies showed that the heterodimer of NFκB bound to the cognate κB-site of the immunoglobulin kappa light chain gene (Baeuerle and Baltimore, 1989, Urban et al., 1991). Further study of the structure of p65 showed it to contain a Rel homology domain (RHD) (Nolan et al., 1991, Ruben et al., 1991). Later, another heterodimer of NFκB was discovered which consisted of Rel B and p50 (Ryseck et al., 1992).

Currently, five subunits of NFκB have been identified; Rel A, Rel B, c-Rel, p52 and p50. In fact, NFκB is not always found as a heterodimer, but also exists as homodimers. Indeed, different combinations of NFκB dimer have been discovered including; p52/c-Rel, p65/c-Rel, Rel A/Rel A, p50/p50, p52/p52 and p50/p52 (Ganchi et al., 1993, Hansen et al., 1994, Kang et al., 1992, Molitor et al., 1990). All NFκB subunits contain a conserved N-terminal region called RHD within which lies DNA-binding and dimerization domains, DNA binding and IκB-binding motifs and a nuclear localisation signal (NLS).

NFκB is further sub-divided in two classes. Class 1 NFκBs consist of p100/p52 and p105/p50. These subunits do not have C-terminal transactivation regions, instead they contain a transrepression domain which consists of a number of ankyrin repeats. Studies have shown that homo- or heterodimers of p50 and p52 can repress κB-dependent transcription in vivo (Brown et al., 1994, Kang et al., 1992, Lernbecher et al., 1993, Plaksin et al., 1993, Schmitz and Baeuerle, 1991). It is believed that the inhibitory effect upon transcriptional activity by p50 and p52 homodimers is due to competition for DNA-binding with other transcriptionally active dimers such as p65/p50 (Plaksin et al., 1993, Schmitz and Baeuerle, 1991).

Rel A, Rel B and c-Rel are classified as Class 2 NFκB subunits. In contrast, these NFκB subunits contain a transactivation domain within the C-terminal RHD. The transactivation domain contains serine, acidic and hydrophobic amino acids. Mutation of any of these amino acids reduces the transactivating activity and transcription of NFκB dependent genes (Dobrzanski et al., 1993). There is an N-terminal leucine zipper-like region in Class 2 NFκB subunits which allow the Class 2 NFκB physically attached to each other. Activation of the C-terminal leads to full activation of most NFκB isoform, however interestingly, both N and C-terminals are required for complete activation of Rel B (Dobrzanski et al., 1993).

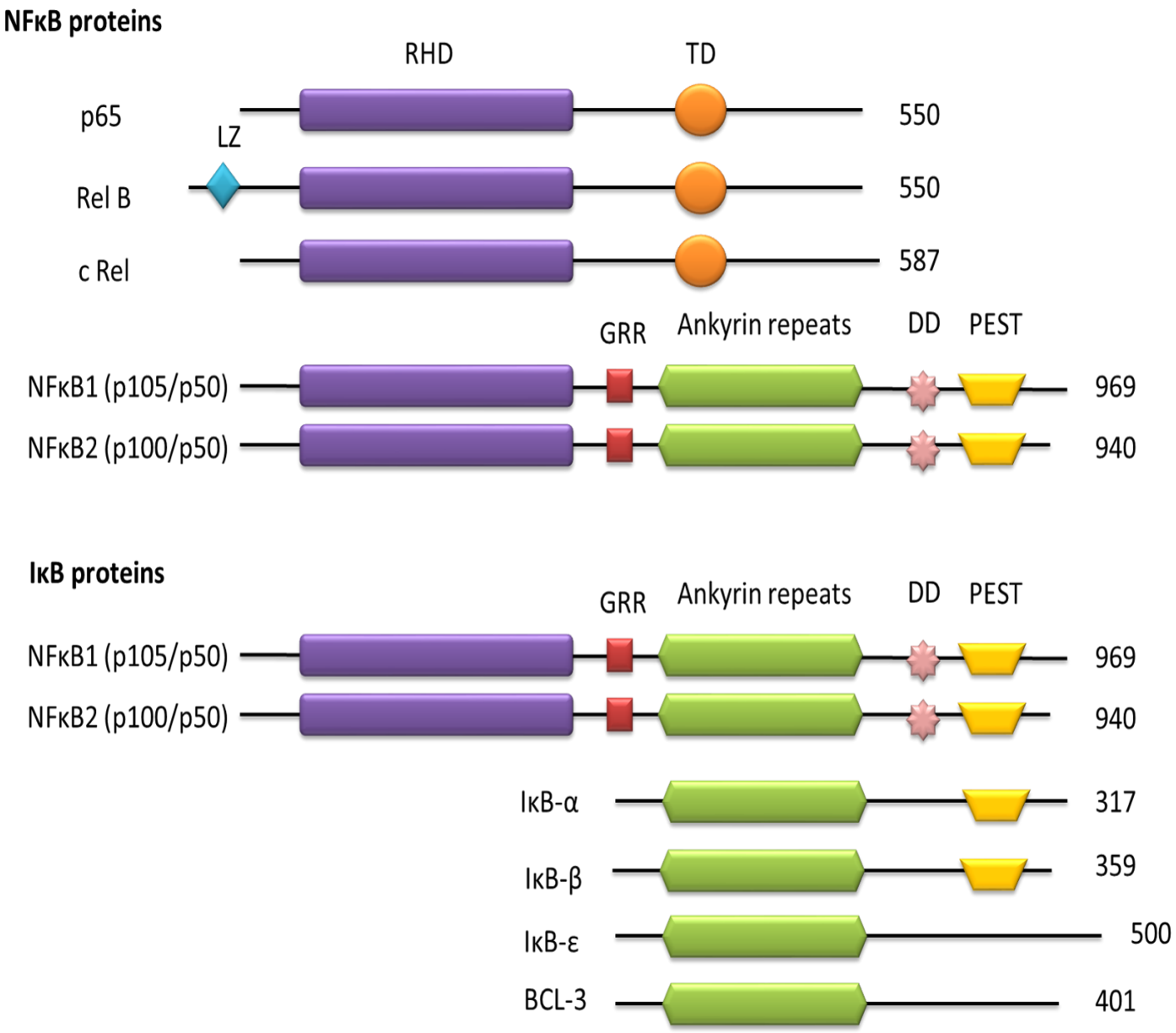


Figure 1.3 NFκB/IκB subunits. The structures of various subunits of NFκB and IκB members. The number of amino acid is shown next to each NFκB and IκB members. LZ, leucine zipper; TD, transactivation domain, Rel homology domain, PEST region, polypeptide sequence enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) region, GRR, glycine-rich region, DD, death domain.

1.3.3.4 Genes Regulated by NFκB

NFκB plays a key role in a regulation of a number of cellular functions including growth, survival, apoptosis, differentiation and development. This in turn is mediated by transcriptional regulation of a number of NFκB dependent genes following binding of homo or heterodimer to specific DNA binding motifs.

For p65/p50, the consensus sequence 5'-GGGRNYYYCC-3' (where R=A or G; Y=C or T; W=T or A; V=A, C or G) (Chen and Ghosh, 1999, Chen et al., 1998), has been identified. Through this sequence, Rel A regulates expression of genes such as ICAM-1, VCAM-1 and COX-2 (Denk et al., 2001). Rel A (p65) is also responsible for the expression of anti-apoptotic genes, for instance X-linked inhibitor of apoptosis protein (XIAP) and cellular inhibitor of apoptosis protein (c-IAP) (Chu et al., 1997, Stehlik et al., 1998b) and pro-survival factors such as bcl-2 (Catz and Johnson, 2001). These genes are responsible for regulation of cell survival and proliferation.

In contrast to Rel A (p65), Rel B is responsible for lymphoid development, immune cell trafficking and angiogenesis. As the genes regulated by Rel B are clearly different than Rel A, unsurprisingly, the DNA binding sequence of Rel B is also distinct. The DNA binding sequence of Rel B has been identified as 5'-GGGRVWTTYY-3' (Britanova et al., 2008). In 2002, Dejardin and co-workers discovered that Rel B controls the expression of several chemokines such as secondary lymphoid tissue chemokine (SLC), B lymphocyte chemoattractant (BLC), EBI-1-ligand chemokine (ELC) and stromal cell-derived factor-1α (SDF1). These genes are responsible for regulating the lymphoid organogenesis process (Dejardin et al., 2002). Wharry and co-workers found genes such as cytokine B cell activation factor (BAFF) and chemokines including chemokine C-X-C motif ligands (CXCL12, CXCL13), C-C motif ligands (CCL19 and CCL21) are up-regulated by constitutive activation of the non-canonical pathway in pancreatic ductal adenocarcinoma (PDAC). Several of these genes are strongly related to the development of lymphoid malignancies and solid tumours (Wharry et al., 2009).

1.3.3.5 Introduction to the NFκB signalling pathway

As mentioned, five subunits of NFκB have been identified which mediate cellular activation of the pathway in the form of specific NFκB dimers with DNA binding and transactivation activity. There are three main signalling pathways which mediate NFκB activation. These are: 1) the canonical NFκB pathway also known as the classical pathway, which is activated by cytokines such as TNF-α and IL-1β, 2) the non-canonical NFκB pathway prevalent in a restricted number of cell types, which is activated by lymphotoxin and 3) the p105 NFκB pathway, which is constitutively active. Figure 1.4 illustrates the mechanisms involved in the activation of each pathway which will be discussed in detail in subsequent sections. For each pathway however, there is a common final mode of activation. At rest, NFκB binds to a corresponding inhibitory molecule called inhibitory kappa B (IκB) which functions to retain NFκB within the cytoplasm. Following cellular activation through a diverse number of signals, including reactive oxygen species (Schreck et al., 1992, Schreck et al., 1991), ultraviolet light (Devary et al., 1993) and pro-inflammatory cytokines, NFκB dissociates from the IκB to allow translocation into the nucleus. In turn, IκB is regulated by a number of upstream mechanisms which will be discussed in the following section.

1.3.3.6 Structure of IκB

In 1988, Baeuerle and co-workers first identified an inhibitory kappa B protein which acted as a specific inhibitor of NFκB transcriptional activity by binding and retaining NFκB within the cytosol (Baeuerle and Baltimore, 1988). All IκB molecules are now known to contain between three and seven ankyrin repeats and to date, cDNAs encoding eight structurally related members of the mammalian IκB family have been cloned: p100, p105 (Mercurio et al., 1993, Rice et al., 1992), IκB-α (Davis et al., 1991), IκB-β (Thompson et al., 1995), IκB-γ (Inoue et al., 1992a), IκB-ε (Li and Nabel, 1997) and Bcl-3 (Ray et al., 1995). Figure1.3 illustrates the structure of all the IκB members.

IκB molecules require the ankyrin repeats to bind to the regions of the RHD within Rel/NFκB dimer (Cervantes et al., 2009, Hatada et al., 1992). This allows the IκB protein to mask the nuclear localization sequence (NLS), and hence prevent nuclear translocation of Rel/NFκB protein (Beg et al., 1992, Henkel et al., 1992). Moreover, mutation of any of the five regions

within the ankyrin repeats of IκB-β results in loss of binding to NFκB p65 and leads to NFκB translocation to the nucleus and enhanced DNA binding (Inoue et al., 1992b).

A number of studies have demonstrated that separate IκB proteins preferentially inhibit distinct Rel/NFκB protein dimers. For example, IκB-α and IκB-β both strongly bind both c-Rel and Rel A (Baeuerle and Baltimore, 1989, Beg et al., 1992, Thompson et al., 1995). In addition, studies show that other IκB proteins such as the 70kDa form of IκB-γ (Grumont and Gerondakis, 1994) and IκB-ε (Li and Nabel, 1997) also inhibit Rel A/p50 dimers. In contrast, it has been demonstrated that p100 and p105 associate less selectively with NFκB isoforms. They can form inhibitory complexes with p50, p52, Rel A and c-Rel (Mercurio et al., 1993, Rice et al., 1992). Unlike any other subunits, however, Rel B can only bind to p100 and p105, which effectively inhibits p52/Rel B heterodimers formation (Dobrzanski et al., 1995). In each of the three NFκB pathways, the relevant IκB proteins function in overlapping but distinct ways suggesting a variety of functional outcomes.

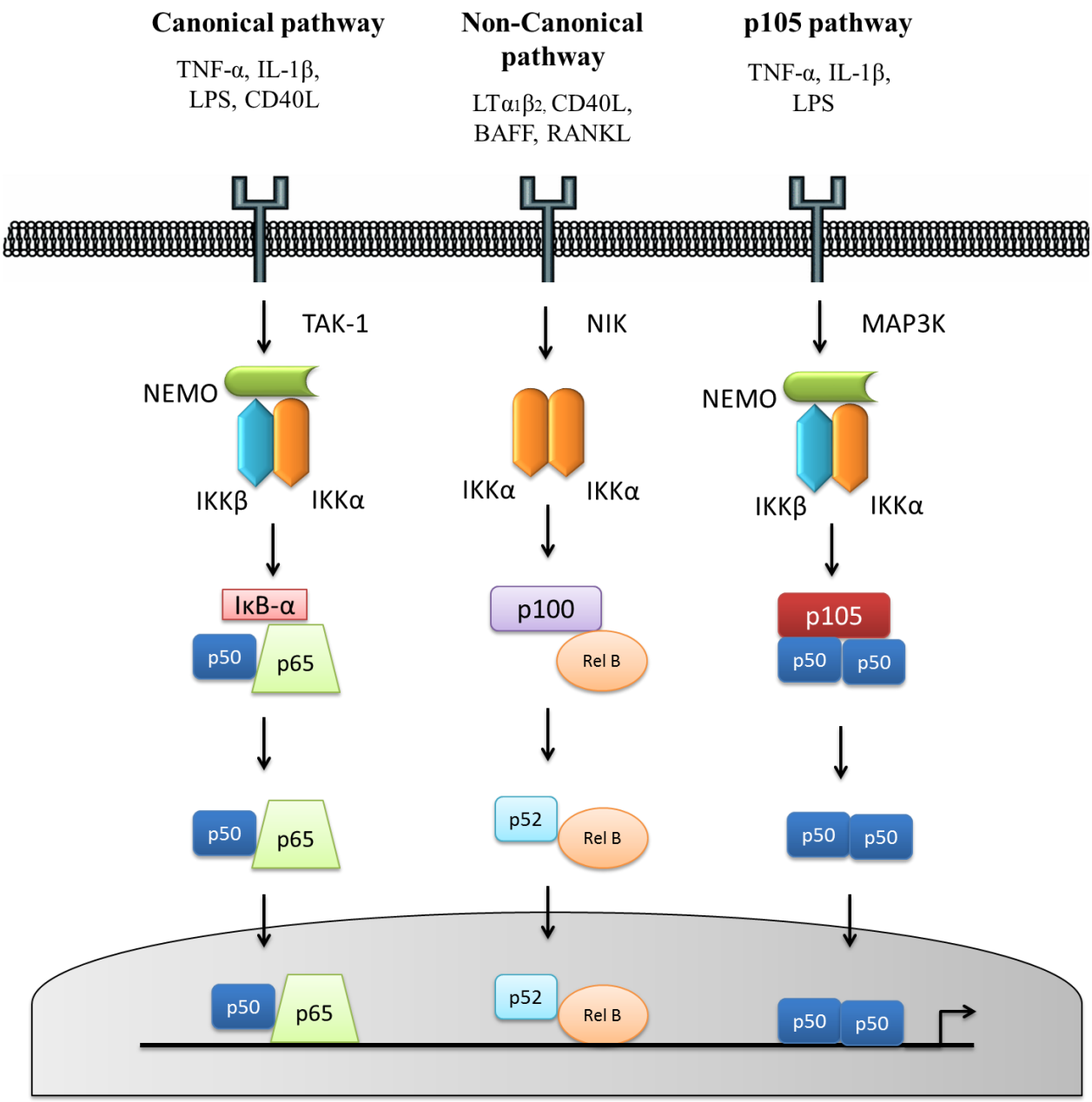


Figure 1.4. Schematic diagram of NFκB pathways. There are three NFκB pathways which are canonical and atypical (non – canonical and p105) pathways. Stimulants bind to various receptors and trigger the signalling pathways, subsequent NFκB dissociated from IκB and translocates into the nucleus to bind to specific DNA sequence and express adhesion molecules and endothelial cytokine (adapted from Beinke and Ley 2004).

1.3.3.6.1 Inhibitory kappa B-alpha (IκB-α)

Two major IκB isoforms were discovered in 1990, which were found to associate with NFκB. These were designated as IκB-α and IκB-β with molecular weights of 37 kDa and 43 kDa respectively (Zabel and Baeuerle, 1990). A number of studies have confirmed that IκB-α but not IκB-β plays an important role in the regulation of the canonical pathway. At rest, IκB-α acts as a specific inhibitor, which binds to the NFκB dimer (p65/p50) to prevent translocation into the nucleus (Jaffray et al., 1995). IκB-α binds to p50 homodimers with the lowest affinity, p65 homodimers with medium affinity, and p50/p65 heterodimers with the highest affinity (Malek et al., 1998). This may be due to more contact sites between IκB-α and p65 than for p50 when bound to the heterodimer (Huxford et al., 1998). Mutation of the NLS site of p65 results in failure of p65/IκB-α binding, this suggests that the p65/ IκB-α binding domain is within the NLS. There are two major regions within the p65 which are important for the binding of IκB-α; aa151 to 312 and aa450 to 500. Mutation within these regions leads to failure of p65/IκB-α binding, but does not modify subsequent events and DNA binding domain enhanced activation (Ganchi et al., 1992).

Following cellular activation, IκB-α is subsequently phosphorylated and ubiquitinated. Serine residues 32 and 36 in IκB-α have been shown to be crucial sites for phosphorylation in response to cellular stimulation by TNF-α, phorbol 12-myristate 13-acetate (PMA) and ionomycin (Brockman et al., 1995, Brown et al., 1993). Phosphorylation of IκB-α functions to initiate ubiquitination and targets the protein for proteasomal degradation which in turn frees NFκB to translocate to the nucleus. Ubiquitination of IκB-α is an essential step before IκB-α degradation (Li et al., 1995). In the same year, it was found that lysine residues 21 and 22 serve as the primary sites for signal-induced ubiquitination of IκB-α, substitution of both residues results in inhibition. With mutation of these sites, IκB-α can still be phosphorylated by the IKK complex (see below) but fails to undergo ubiquitination and degradation, hence NFκB cannot dissociate from IκB-α and remains within the cytoplasm (Chen et al., 1995, Scherer et al., 1995).

1.3.3.6.2 Inhibitory kappa B NFκB2 (p100/p52)

The activation of RelB/p52 is largely distinct from other NFκB subunits and the role of p100 as an IκB member is unique. p100/p52 is also called NFκB2 but was originally named as p98

when characterised as a 98kDa protein. Mercurio and co-workers originally found that the NFκB related protein p55 was produced from p98 via a processing mechanism (Mercurio et al., 1992, Mercurio et al., 1993). Later, this mechanism was designated as p100/p52 processing to reflect the slight difference in the molecular weights of these two particular proteins. Similar to IκB-α, p100 also contains ankyrin repeats in the C-terminal region, whilst the C-terminal domain of both p100 and p105 are required to retain Rel B in cytoplasm. At rest, both p100 and p105 are capable of inhibiting agonist-induced NFκB activation (Mercurio et al., 1993, Rice et al., 1992). The proteolytic processing of p105 and p100 involves proteasome-mediated degradation of the C-terminal half of the protein including the ankyrin repeat domain, resulting in formation of p50 and p52 respectively (Fan and Maniatis, 1991, Siebenlist et al., 1994). Although p100 usually forms a complex with Rel B, in fact, p100 is also capable of interacting with c-Rel. Both p100/c-Rel and p105/c-Rel complexes were found to be processed into active p52/c-Rel complexes (Mercurio et al., 1993). Further details will be discussed in relation to signalling through the non-canonical pathway in Section 1.3.3.9.

1.3.3.7 Identification of Inhibitory Kappa B kinase

As described in Section 1.3.3.6, IκB phosphorylation is essential for activation of the NFκB signalling pathway. However, the kinases responsible for this process were not easily identified and a series of candidates were investigated including PKC isoforms, MAP kinases, MEKKs and PKB. In 1996 Chen and co-workers identified a high molecular mass complex (700-900 kDa) which regulated the phosphorylation of IκB-α at serine 32 and serine 36 (Chen et al., 1996). This complex was identified and later named as IκB kinase (IKK). The functional role of the IKK complex was then elucidated by DiDonato and co-workers in 1997. They first demonstrated that in HT-29 cell, TNF-α activated IKK complexes and then showed over-expression of IKKα induced IκB-α phosphorylation and degradation. Moreover, overexpression of IKK increased TNF-α or IL-1β induced NFκB luciferase activity, while anti-sense IKK completely abolished TNF-α or IL-1β induced NFκB luciferase activity (DiDonato et al., 1997).

The IKK complex consists of IKKα, IKKβ and IKKγ. Both IKKα and IKKβ are catalytically active kinases. An early study showed IKKα and IKKβ to have similar structures with the overall protein sequence of IKKα and IKKβ being 52% identical. Both proteins have an NH_2-

terminal kinase domain sharing 64% identity, a C-terminal helix-loop-helix (HLH) domain and a leucine-zipper motif located in the centre of the kinase which is involved in homodimer and heterodimer formation (Woronicz et al., 1997). Also called NFκB-essential-modifier (NEMO), IKKγ is a non-catalytic regulatory component. An amino-terminal α-helical region of NEMO is responsible for binding to the carboxyl-terminal of either IKKα or IKKβ. Blocking the association of NEMO with the IKKs leads to inhibition of cytokine-induced NFκB activation and hence the genes regulated by NFκB (May et al., 2000). Studying the role of individual IKK subunits is important to broaden the knowledge of NFκB signalling pathways and this has been a topic of intense study over the last 15 years.

1.3.3.8 The canonical (classical) NFκB pathway

Concomitant with the discovery of the IKKs as key kinases involved in the regulation of NFκB activation was the identification of intermediates which link initial cellular activation to IKK itself. This was the case in particular for cytokine receptors, for example TNFR1 and the IL-1β receptor as well as the toll-like receptors (TLRs) which were found to mediate the action of both Gram positive and Gram negative bacteria and other infectious agents. These receptors are well recognised to activate the NFκB pathway in a large number of cell types (Plotnikov et al., 2011).

In response to TNF-α, the IKK complex is recruited to TNFR1 via TNF receptor-associated factor 2 (TRAF2) and then activated. The interaction occurs through the leucine zipper motif of IKKα, IKKβ and the RING finger domain of TRAF2 (Devin et al., 2001). In support of this model, Rothe and co-workers showed that over-expression of TRAF2 results in increased p65-DNA binding and also NFκB-driven luciferase reporter activity through activation of the other TNF-α receptor isoform, TNFR2. Furthermore, HEK 293 cells containing mutated TRAF2, which lacks the NH_2 terminal 86 amino acids that comprise the RING finger domain, inhibited TNF-α stimulated NFκB luciferase activity (Rothe et al., 1995). Transforming growth factor β-activated kinase 1 (TAK-1) is a member of the MAP3K family which also plays a critical role in activation of the IKK complex through phosphorylation. TNF-α induces a rapid association of TAK1 with IKKα and IKKβ and also TRAF2. Moreover, siRNA directed against TAK1 inhibits TNF-α induced IκB-α degradation and hence the activation of NF-κB pathway (Takaesu et al., 2003).

In contrast, TRAF6 but not TRAF2 is required for IL-1β induced NFκB activation. In response to IL-1β stimulation, an additional intermediate, TAB2, translocates from the membrane to the cytosol and acts as an adaptor to link TAK-1 with TRAF6. (Takaesu et al., 2000). A recent study showed TAK-1 binding protein 2 (TAB2) facilitates ubiquitination of TRAF6 in IL-1 stimulated cells (Kishida et al., 2005). Kanayama and co-workers found TAB2 and TAB3 bind preferentially to lysine 63-linked polyubiquitin chains of TRAF6 through a highly conserved zinc finger (ZnF) domain which subsequently leads to ubiquitination of the IKK complex, while mutation of the ZnF domain abolishes the ability of TAB2 and TAB3 to bind to polyubiquitin chains and hence fails to activate TAK1 and the IKK complex (Kanayama et al., 2004). Subsequently activated TAK1 phosphorylates the IKK complex and the downstream pathway (Wang et al., 2001).

Interestingly it has been demonstrated that there is a negative feedback mechanism in the canonical NFκB pathway which involves the deactivation of IKKβ. When pro-inflammatory agents such as IL-1 activate the canonical pathway, free NFκB translocates to the nucleus to regulate the expression of specific genes one of which is IκB-α. While IκB-α is resynthesised, it is then exported from the nucleus back to cytoplasm and binds to NFκB to form an inactive complex again. In parallel, protein phosphatase 2A (PP2A) dephosphorylates IKKβ that allows for stabilization of resynthesized IκB-α in cytoplasm (Barisic et al., 2008).

1.3.3.8.1 The role of IKKβ in the regulation of canonical NFκB pathway

The initial identification and purification of the IKK complex indicated that IKKβ played a critical role in the canonical NFκB pathway. However subsequent studies were required to confirm this model and involved the use of IKKβ knockout mice, cellular expression of dominant-negative mutants (DN) of IKKβ and more recently siRNA approaches.

Early work revealed difficulties in generating IKKβ knockout mice for studies both *in vitro* and *in vivo* as IKKβ knockout embryos died at approximately at day E 12.5. Histopathological examination revealed the cause of death to be massive liver apoptosis (Li et al., 1999c, Li et al., 1999d). Interestingly, an earlier study indicated that p65 and p50 knockout mice also died at E12.5 (Horwitz et al., 1997), implicating IKKβ in the canonical NFκB signalling. Despite these difficulties, it was shown that in IKKβ deficient mouse embryonic fibroblast (MEFs), there was a marked reduction in TNF-α and IL-1α induced

NFκB activity (Li et al., 1999c) and as a consequence, enhanced apoptosis in response to TNF-α (Li et al., 1999d).

Later, additional experiments confirmed that IKKβ is crucial in regulating NFκB in various cell types. Overexpression of wild type IKKβ in HEK239 cells led to phosphorylation on serine residues 32 and 36 of IκB-α and serines 19 and 23 of IκB-β and activation of NFκB, whilst conversely, overexpression of a DN-IKKβ (K44A) (deficient for ATP binding) inhibited cytokine induced NFκB activation (Woronicz et al., 1997). TNF-α induced IκB-α degradation was inhibited in MEFs from IKKβ knockout mice which also failed to response with an increase in NFκB activity following IL-1α stimulation (Solt et al., 2009). Catley and co-workers showed that DN-IKKβ inhibited either TNF-α-or IL-1β-induced NFκB-DNA binding and NFκB linked luciferase activity in human pulmonary epithelial A549 cells (Catley et al., 2005). Moreover, TNF-α induced IκB-α degradation was inhibited by DN-IKKβ kinase adenovirus which resulted in abolition of NFκB activity in HUVECs (Denk et al., 2001) and vascular smooth muscle cell (VSMC) (MacKenzie et al., 2007).

Importantly, a number of these studies also demonstrated that IKKβ can also mediate phosphorylation of NFκB p65 to initiate transactivation, leading to increased transcriptional activation following DNA binding. DN-IKKβ adenovirus inhibited NFκB p65 phosphorylation at serine 536 in HUVECs (Denk et al., 2001), with a similar result obtained in vascular smooth muscle (MacKenzie et al., 2007) and HeLa cells (Sakurai et al., 1999).

Significantly, many of these studies also linked IKKβ to the regulation of a number of cellular responses including, inflammation and cell survival through the activation of p65 NFκB dependent gene transcription. It came as little surprise to discover that the inhibition of IKKβ prevented the expression of genes important in these events. Again following DN-IKKβ expression, Denk and co-workers found that TNF-α failed to induce ICAM-1, VCAM-1, MCP-1, E-selectin and COX-2 expression (Denk et al., 2001). Similarly, MacKenzie and co-workers also showed that IKKβ was crucial in the regulation of ICAM-1 and VCAM-1 expression in vascular smooth cells (MacKenzie et al., 2007). Furthermore, inhibition of IKKβ kinase activity also led to inhibition of inflammatory genes expression such as IL-8, COX-2 and ICAM-1 in A549 cell (Catley et al., 2005). This evidence also links IKKβ to a number of diseases states such as arthritis and cancers and this will be examined in subsequent sections.

1.3.3.8.2 The role of IKKα in the regulation of the canonical NFκB pathway

Although IKKα and IKKβ share 64% identity in the NH_2-terminal kinase domains as mentioned in Section 1.4.2, the functional role of IKKα within the canonical pathway remained obscure. As with IKKβ, early studies again showed difficulty in developing IKKα knockout mice. Similar to IKKβ $^{-/-}$ mice, global knockout of IKKα also resulted in embryonic lethality. However, the phenotype of IKKα$^{-/-}$ embryos was completely different from that of IKKβ $^{-/-}$ at the same developmental stage. Furthermore IKKα$^{-/-}$ mice were born and lived for 30 minutes. The rudimentary limb and tail, a large omphalocele, and craniofacial features were all deformed in new-born IKKα$^{-/-}$ mice. The most obvious phenotype was the skin which was taut, shiny, and completely devoid of wrinkles (Hu et al., 1999). Other studies suggested that skin abnormality in IKKα$^{-/-}$ mice could be attributable to dysregulation of keratinocyte differentiation and proliferation (Hu et al., 1999, Li et al., 1999b, Takeda et al., 1999). Neither IKKβ $^{-/-}$ nor IKKγ $^{-/-}$ mice showed a similar skin phenotype, which suggested that IKKα had a role independent from the canonical NFκB pathway.

More recent experiments have further confirmed an essential role for IKKα in skin function. IKKα controls the production of a soluble factor such as keratinocyte differentiation-inducing factor (KDIF) that induces keratinocytes differentiation (Hu et al., 2001). Later, Liu and co-workers found a marked reduction in IKKα expression in poorly differentiated human squamous cell carcinomas (SCC) and identified IKKα mutations in exon 15. It is believed that the activity of IKKα in the epidermis may be responsible for resistance to chemical carcinogen-induced mitogenic and angiogenic activities, repressing tumour progression and metastases, thus lack of IKKα may lead to development of human skin cancers (Liu et al., 2006). In addition, IKKα was found to be essential for mammary gland development during pregnancy. A low level of cyclin D1 expression leads to impaired proliferation of mammary epithelial cells in IKKα$^{-/-}$ mice (Cao et al., 2001). The abnormality is due to the inability of mammary epithelial cells to respond to receptor activator of NFκB (RANK) ligand, which activates NFκB-induced cyclin D1 expression (Cao et al., 2001).

The distinctive phenotype of the IKKα knockout mice/cells relative to the knockout IKKβ and their suggested different functional roles has been supported by several types of cellular study. Initial finding confirmed that IKKα is not required for upstream activation of the canonical NFκB pathway by pro-inflammatory stimuli. TNF-α, IL-1β and LPS can still

activate the IKK complex to induce IκB-α degradation and p65 nuclear translocation in IKKα$^{-/-}$ MEF, primary keratinocytes and liver tissue (Hu et al., 1999). Similar results were achieved in different cell types, inhibiting IKKα kinase activity did not affect TNF-α induced IκB-α degradation in HUVECs (Denk et al., 2001), vascular smooth muscle cells (MacKenzie et al., 2007) and MEFs (Solt et al., 2009). In addition, Yang and co-workers demonstrated that deletion of IKKα did not affect LPS-induced p65 phosphorylation in MEFs (Yang et al., 2003). Interestingly, contradictory studies showed that both TNF-α and IL-1β induced NFκB/DNA binding could be reduced in IKKα$^{-/-}$ MEFs (Hu et al., 1999, Solt et al., 2009), while, Catley and co-workers demonstrated that inhibiting IKKα kinase activity through expression of a K44A inactive mutant resulted in a failure to induce NFκB driven luciferase reporter activity in response to TNF-α or IL-1β human pulmonary A549 cells (Catley et al., 2005). Taken together, these studies suggested the potential of a cell type dependency in regulation of the NFκB pathway by IKKα.

A number of studies have shown that despite IKKα playing no role in regulating p65 nuclear translocation and DNA binding, the expression of several p65 dependent genes could be regulated by IKKα including MCP-1, IL-8, ICAM-1, VCAM-1, E-selectin and cIAP-2. (Denk et al., 2001, Gloire et al., 2007, Huang et al., 2007). These results suggested the potential for IKKα to regulate the canonical NFκB pathway at a site downstream of the IKK/IκB-α/p65 complex. In 2003, Anest and co-workers discovered TNF-α induced IKKα translocation into the nucleus from the cytoplasm (Anest et al., 2003). They showed that a requirement for IKKα in mediating H3 Serine 10 phosphorylation at different NFκB-dependent promoters such as IκB-α and IL-6 (Anest et al., 2003). Another study confirmed a role for IKKα cytokine-induced phosphorylation and subsequent acetylation of specific residues in histone H3 and in addition, revealed that IKKα interacts with CREB-binding protein (CBP) within the nucleus and in conjunction with p65, is recruited to IκB-α and IL-8 promoters (Yamamoto et al., 2003). Later, Huang and co-workers found that the IKKα can also down-regulate the expression of p53 dependent genes. They found IKKα-mediated CBP phosphorylation not only switches the binding preference of CBP from p53 to p65, but also causes redistribution of CBP recruitment from p53 responsive to p65 responsive promoters, thereby enhancing NFκB dependent genes expression such as ICAM-1 and cIAP-2 (Huang et al., 2007).

Other more recent studies have implied that some of the actions of IKKα may be independent of kinase activity. In IKKα$^{-/-}$ MEFs, reconstitution with DN-IKKα allows gain of function for a subset of genes. Relative to wild type IKKα, this subset was small but nevertheless suggest that IKK may function as a scaffold protein (Li et al., 2002). Similar data was obtained by Massa and co-workers, expression of a kinase inactive IKKα mutant (K44M) in IKKα$^{-/-}$ MEF rescued expression of approximate 28% of NFκB genes (Massa et al., 2005). These observations can also be found in other cell types, Hu and co-workers showed that IKKα regulates the secretion of an autocrine factor that induces keratinocyte differentiation, independent of its kinase activity (Hu et al., 2001). Overall, the studies outlined above confirmed a role for IKKα in the regulation of the canonical pathway but indirectly and at the level of p65-dependent gene transcription.

1.3.3.8.3 The role of IKKγ in the regulation of canonical NFκB pathway

Whilst the function of IKKα and β have been studied in great detail, IKKγ has been examined to a much lesser extent. IKKγ was purified and found to have an overall protein structure somewhat different from either IKKα or IKKβ. Molecular cloning and sequencing indicated that IKKγ contains several potential coiled-coil motifs (Rothwarf et al., 1998). IKKγ is a 48kDa non-catalytic protein, as described above; it plays a regulatory role and is required for IKK complex activation (Rothwarf et al., 1998, Yamaoka et al., 1998). Early studies showed mutation within the C-terminal results in failure to activate the IKK complex and hence NFκB activity (Rothwarf et al., 1998). Later, May and co-workers found that IKKγ interacts with a domain within IKKβ, designated the NEMO-Binding Domain (NBD), to regulate the formation of the IKK complex (May et al., 2000, May et al., 2002). More recent evidence suggests that the NBD is essential for IKK complex activation and regulation of canonical NFκB activity. Furthermore, a mutation in the NBD results in failure to activate the canonical NFκB pathway (Solt et al., 2009).

In contrast, mutating the NBD of IKKγ to prevent interaction with IKKα does not affect p100 processing, thus implicating that the association of IKKα and IKKγ is not involved in regulation of the non-canonical pathway (Solt et al., 2009). Activation of the IKK complex as a component of up-stream signalling required ubiquitination of IKKγ. A CBM complex consisting of the proteins CARMA1, Bcl10 and MALT1 is thought to be important in mediating ubiquitination of IKKγ. Following activation of the TNF-α or IL-1β receptor,

Bcl10 is conjugated with K63-linked ubiquitin chains and associates with IKKγ (Wu and Ashwell, 2008). This mechanism allows phosphorylation of IKKα/β and subsequent translocation of p65/p50 into the nucleus.

1.3.3.9 The up-stream activation of the Non-Canonical NFκB pathway

Concomitant with analysis of the classic canonical pathway is the study of IKKα and β in the regulation of other NFκB pathways. The non-canonical pathway is classified as one of these atypical pathways prevalent in a restricted number of cell types and is characterised by p100/p52 processing. Figure 1.4 shows the important kinases and proteins involved in the pathway and some of the cognate agonists involved. The non-canonical signalling pathway is activated by TNFR superfamily members, including B cell-activating factor receptor (BAFFR) (Claudio et al., 2002, Kayagaki et al., 2002), CD40 (Coope et al., 2002), lymphotoxin β receptor (LTβR) (Dejardin et al., 2002), RANK (Novack et al., 2003), TNFR2 (Rauert et al., 2010). An early study showed that Receptor activator of nuclear factor kappa-B ligand (RANKL) can activate the non-canonical NFκB pathway through processing of p100 to p52 through RANK in osteoclast precursors (Novack et al., 2003) to control differentiation (Teitelbaum, 2000). Both TNF receptor-associated factor 2 (TRAF2) and TNF receptor-associated factor 3 (TRAF3) are recruited to the receptor complex following ligand ligation (Bishop and Xie, 2007), then both TNAF2 and TNAF3 undergo degradation which leads to activation of NIK and the further downstream IKKα itself. Similar to TAK-1, NFκB-inducing kinase (NIK) is also a MAP3K member which activates the IKK complex but in particular, IKKα dimers, to subsequently regulate p100 processing (Xiao et al., 2001; Senftleben et al., 2001). Ling and co-workers showed that NIK phosphorylated IKKα at residue serine-176. A mutated form of IKKα containing alanine at residue 176 could not be phosphorylated or activated by NIK and inhibited IL-1 or TNF-α-induced NFκB activation (Ling et al., 1998).

NIK itself may further regulate p100 processing through ubiquitination (Xiao et al., 2001). p100 contains a region called the NIK-responsive domain (NRD). Substrate-binding subunit $SCF^{\beta TrCP}$ is an ubiquitin ligase complex and the βTrCP component of the ligase complex binds to the NRD site of p100 to induce ubiquitination (Fong and Sun, 2002). Later, scientists identified amino acid lysine-856 within the substrate binding subunit of p100 as crucial in the ubiquitination and processing of p100 (Amir et al., 2004).

It should also be noted that downstream intermediates can also regulate upstream components of the non-canonical pathway. For example, IKKα phosphorylates NIK and attenuates the stabilisation of NIK, resulting in a decrease in non-canonical NFκB signalling. It is believed that IKKα destabilises NIK to prevent over activation of non-canonical NFκB signalling (Razani et al., 2010). The mechanisms underpinning IKKα negative feedback regulation of Rel-B dependent gene expression and the involvement of the other upstream mediators of the non-canonical pathway are not yet fully understood, but it is possible that the phosphorylation of NIK can be considered a biomarker of IKKα activation.

1.3.4 The role of IKKα in the regulation of the Non-Canonical NFκB pathway

A number of studies using deletion mice (Luftig et al., 2004, Muller and Siebenlist, 2003) or other approaches clearly demonstrated a role for IKKα in the non-canonical pathway and at the same time excluded the involvement of IKKβ (Claudio et al., 2002, Dejardin et al., 2002, Liang et al., 2006, Senftleben et al., 2001). In addition, no role for NEMO was identified, suggesting a lack of requirement for the classical IKK complex. As mentioned above, activation of this pathway requires both ubiquitination and phosphorylation of p100, and indeed Liang and co-workers found that IKKα phosphorylates p100 within residues serine 866 and serine 870 and this is crucial for p100 processing (Liang et al., 2006). Following expression of a dominant-negative isoform of IKKα, p100 processing is inhibited and p52 formation is reduced (Luftig et al., 2004, Muller and Siebenlist, 2003). Therefore, IKKα is essential for the formation of the Rel B/p52 heterodimer which subsequently translocates into the nucleus to mediate gene transcription.

A number of studies also link IKKα to a specific subset of genes. There are several studies showing that inhibiting IKKα can affect Rel B dependent genes expression. Dejardin and co-workers showed that in MEFs inhibiting IKKα activity leads to reduction in expression of both chemokine and cytokine genes including SLC, BLC, ELC, SDF-1 and BAFF (Dejardin et al., 2002). In addition, Bonizzi and co-workers confirmed that IKKα is required for activation of the non-canonical NFκB signalling pathway again based on p100 processing. They also found that the promoters of IKKα dependent genes are recognised by Rel B/p52 dimers but not by p65/p50 (Bonizzi et al., 2004). Later, it was shown that IKKα regulates the cell cycle gene S-phase kinase-associated protein (skp2) which is RelB/p52 dependent

(Schneider et al., 2006). These results provided strong evidence for a crucial role for IKKα in the regulation of non-canonical NFκB signalling pathway.

More recent studies have identified other functions of IKKα particularly in relation to cell cycle regulation and apoptosis. IKKα has been shown to regulate cyclin D1 but the findings are contradictory. On the one hand, a number of studies demonstrate that IKKα and Rel B/p52 axis up-regulates the expression of cyclin D1 (Cao et al., 2001, Demicco et al., 2005). On the other hand, other studies have shown that IKKα down-regulates cyclin D1 expression. Song and co-workers also reveal that IKKα plays a critical role in mediating UVB-induced G0/G1 cell cycle arrest by suppressing cyclin D1 expression in MEFs (Song et al., 2010). The mechanism of IKKα induced cyclin D1 degradation has been investigated by Kwak and co-workers. They show that IKKα can regulate the subcellular localization and proteolysis of cyclin D1 by phosphorylation at Threonine-286. (Kwak et al., 2005).

A recent study has not uncovered the potential for Aurora-A to be regulated by IKKα. Aurora-A is a member of mitotic kinases that localise to centrosomes. Aurora-A kinases have been implicated in centrosome separation and spindle assembly (Bischoff and Plowman, 1999, Giet and Prigent, 1999, Nigg, 2001). The activity of Aurora A kinase peaks during the G_2/M phase and coincides with phosphorylation of IKKα (Bischoff and Plowman, 1999). IKKα facilitates the regulation of M phase of the cell cycle by modulating Aurora A phosphorylation and activation (Prajapati et al., 2006). Further studies are required to fully characterise the independence of NFκB regulation and signaling of IKKα.

1.4 The role of Non-Canonical NFκB pathway in regulation of inflammation

A substantial number of studies implicate a role for the canonical NFκB pathway and IKKβ in the regulation of inflammation and these are examined in the context of diseases such as arthritis, inflammatory bowel disease and atherosclerosis (see Section 1.5). Far fewer studies have examined a role for IKKα. Nevertheless, indirect evidence is now accumulating to link IKKα with the negative regulation of acute inflammation but with a positive role in chronic inflammation through an interaction with the adaptive immune system.

With identification of the TLRs, a large number of studies have conclusively established an important role for the IKKβ/IκB-α/p65 axis (NFκB canonical signaling pathway) in response

to a vast array of infectious agents. In contrast, a recent study has shown that IKKα activation limits the inflammatory response to bacterial infection *in vivo* and inhibits activation of NFκB in primary macrophages *in vitro* (Lawrence et al., 2005). Macrophages from transgenic mice expressing catalytically-inactive IKKα were found to be resistant to pathogen-induced apoptosis, due to elevated expression of anti-apoptotic genes, and showed increased expression of pro-inflammatory cytokines such as TNF-α (Lawrence et al., 2005). In cellular studies, IKKα has been found to negatively regulate the canonical NFκB pathway and indeed, IKKα knockout macrophages derived from foetal liver stem cells showed an increase level of p65 dependent pro-inflammatory cytokines production and an enhanced ability to stimulate T-cell proliferation (Li et al., 2005). Similarly, another research team also demonstrated increased expression of pro-inflammatory cytokines and chemokines upon stimulation with LPS in IKKα knockout fibroblasts (Xia et al., 1997). Interestingly, the results of IKKα knockout studies can sometimes be different from those utilising DN-IKKα. For instance, the activity of IKKβ is elevated in IKKα knockout cells, but this cannot be observed in cells overexpressing DN-IKKα (Lawrence et al., 2005). This may be due to the formation of an IKKβ homodimer with increased NFκB activity which only occurs in absence of the IKKα protein.

In general, studies showing a direct role for IKKα in other aspects of inflammation linked to immune function are lacking, however some information has accumulated via the study of Rel B function, which is downstream of IKKα. Rel B-deficient mice died of multi-organ inflammation (Weih et al., 1995, Xia et al., 1997), due to autoreactivity of endogenous T-cells, subsequently causing abnormal development of the thymus and the breakdown of immunological tolerance. Rel B has also been shown to inhibit IκB-α stability and therefore limit NFκB activation (Xia et al., 1999). Moreover, Rel B may negatively regulate canonical NFκB complexes in the nucleus through protein-protein interactions with Rel A (Jacque et al., 2005). At present, it is unclear how these cellular studies link to *in vivo* affects within their immune system.

Overall it is accepted that the non-canonical NFκB pathway plays an important role in the regulation of immunity. Deletion of Rel B and NFκB-2 inhibits the ability of the antigen presenting cell (APC) to activate T cells in defending against foreign bacteria and virus (Gerondakis et al., 1999). Moreover, a Rel B deficiency leads to immune failure and uncontrolled spread of the vaccinia virus in lung and liver (Freyschmidt et al., 2007). The

activators of the TNF superfamily, CD40 ligand, RANKL or osteoclast differentiation factor (ODF), are expressed by the activated T cell. Their interaction with receptors on APC including CD40 and RANK, subsequently induces Rel B activation that promotes survival and augments the ability of the APC to simulate T cell proliferation and activation, conceivably by up-regulating the expression of NFκB-dependent molecules MHC class 2, CD80, and CD86 (Anderson et al., 1997, Li et al., 1999a, Verhasselt et al., 1999, Wong et al., 1999).

1.5 NFκB and inflammatory diseases

Rheumatoid arthritis is a chronic, systemic inflammatory disease causing joint destruction, and is associated with increased morbidity and mortality (Firestein, 2003, McGorisk and Treasure, 1996, Smolen and Steiner, 2003). Evidence suggests that NFκB can play a central role in many aspects of the disease. Activated NFκB has been detected in human synovial tissue during the early stage of joint inflammation (Gilston et al., 1997). Moreover, the p65/p50 complex was found to be located in the endothelium and synovial lining of rheumatoid arthritis patients, particularly in CD14-positive cells, but not in the normal synovium (Handel et al., 1995).

In fact, clinical studies outlined above have been supported by a number of cellular animal studies utilising both pharmacological and molecular studies. Han and co-workers found increased synovial NFκB-DNA binding precedes the development of joint involvement in murine collagen-induced arthritis (CIA) and gradually increases during the evolution of disease (Han et al., 1998). In addition, synovial NFκB activation also occurs within a few days after immunization in rat adjuvant arthritis (Tsao et al., 1997). Furthermore, an intra-articular injection of Adv. DN-IKKβ reduces nuclear translocation of NFκB (p65/p50) and clinical synovitis in adjuvant arthritis in rats (Tak et al., 2001). However, inhibiting IKKβ also has the potential to worsen the inflammatory condition by reducing the synthesis of anti-inflammatory agents such as cyclopentenone prostaglandins (Rossi et al., 2000) and COX-2 (Chang et al., 2004). Evidence suggests that COX-2 also has anti-inflammatory effect (Gilroy et al., 1999).

Dysregulated cytokine production and signalling mechanisms by intestinal epithelial cells, lymphocytes and macrophages have been implicated in the pathogenesis of inflammatory

bowel disease (IBD). An early study showed there was an increase of NFκB expression in mucosal macrophages from IBD patients due to the over-expression of the cytokines such as TNF-α, IL-1β and IL-6 (Neurath et al., 1996). Furthermore, NF-κB-induced TNF-α was in turn able to potentiate the activation of NF-κB, thereby providing a kind of positive feedback in these cells (Hart et al., 1998, Holtmann and Neurath, 2004). High cellular NFκB activity was also observed in gastric epithelial cells under the pathogenic condition of helicobacter pylori-associated gastritis (van Den Brink et al., 2000). Similarly, a clinical study also showed high levels of NFκB DNA-binding activity accompanied by an increased production of IL-1, IL-6 and TNF-α in lamina propria macrophages during Crohn's disease (CD) and ulcerative colitis (UC). Moreover, the expression of NFκB isoforms including p50, p65 and c-Rel was found to be dramatically high in patients with CD and UC. However, the production of pro-inflammatory cytokines was remarkably reduced using specific antisense phosphorothioate oligonucleotide to down-regulate p65 expression in macrophages during inflammatory bowel disease.(Neurath et al., 1998).

NFκB also plays an important role in several other tissue specific models of inflammation. Activation of NFκB leads to production of pro-inflammatory cytokines and adhesion molecules in nephritic glomeruli. Therefore uncontrolled NFκB regulation may cause the development of glomerulonephritis. A recent study showed that using a potent NFκB inhibitor, pyrrolidine dithiocarbamate (PDTC), glomerular NFκB-DNA binding activity was reduced and subsequently caused reduction of IL-1β, MCP-1, ICAM-1 and iNOS mRNA expression (Sakurai et al., 1996). PDTC also prevented urinary protein excretion which is a pathophysiological parameter for glomerulonephritis (Sakurai et al., 1996). It was also demonstrated that c-Rel knockout mice have decreased airway hyperreponsiveness and eosinophil infiltration as well as lower levels of serum immunoglobulin (IgE) in an allergen-induced asthma model (Donovan et al., 1999). Moreover, inflammatory airway disease in humans was found to be strongly related to the expression of cytokine and adhesion molecules elevated due to the activation of NFκB. Furthermore high levels of nuclear NFκB were observed especially in airway epithelial cells and led to an increased expression of pro-inflammatory cytokines, chemokines, iNOS and COX-2 (van Berlo et al., 2010).

Overall these observations support the notion that NFκB plays an important role in many different inflammatory disorders. However, the vast majority of studies implicate only IKKβ

in these mechanisms and additional studies are required which focus on the non-canonical IKKα axis.

1.6 Atherosclerosis

Atherosclerosis is a chronic inflammatory condition of the blood vessels. The condition is associated with plaque formation in the sub-endothelial layer, denudation of the endothelial layer and thrombus formation. Subsequent destabilisation of the plaque and rupture leads to blockage of vessels and result in adverse cardiovascular events, heart attack and stroke. This process is initiated by migration of monocytes into the subendothelial space. Monocytes differentiate into macrophages and eventually become lipid-laden foam cells due to take up of modified LDL via lipoprotein molecules circulating in the blood stream. Many studies suggest Apolipoprotein E (ApoE) prevents atherosclerosis by clearing cholesterol-rich lipoproteins from plasma. Under physiological condition, ApoE binds to the cell surface low-density lipoprotein (LDLR) or LDLR-related protein (LRP) and initiates receptor-mediated endocytosis to prevent cellular uptake of LDL (Mahley and Rall, 2000). Reducing the expression of ApoE in human patients has been found to be associated with proatherogenic lipoprotein profile and atherosclerotic disease development (Ghiselli et al., 1981, Schaefer et al., 1986). Lipoprotein accumulation, matrix degradation and monocytes migration are the major factors that initiate the atherosclerotic plaque development (Saxena and Goldberg, 1994).

1.6.1 Development of atherosclerosis

The endothelial cell plays a key role in the development of atherosclerosis. These cells respond to a number of pro-inflammatory cytokines including TNF-α and IL-1β to increase the expression of inflammatory mediators such as IL-6, COX-2, ICAM-1, VCAM-1 and MMPs. Furthermore, there is a concomitant reduction in the release of protective mediators such as PGI2 and NO. Therefore, control of the inflammatory status of endothelial cells, which is achieved by a balance of pro- and anti - inflammatory signals, is crucial to limiting the disease (Hoefen and Berk, 2002). Initiation of atherosclerosis also involves the release of oxygen radicals and lipid mediators which can be susceptible to peroxidation and can also activate endothelial cells (Reddy et al., 2002). LDL which accumulates within the vessel wall is also subject to oxidation which allows endothelial cell activation (Reddy et al., 2002). These events are also related to a number of other diseases of the cardiovascular system

including diabetes. These conditions are always associated with an increase in endothelial dysfunction leading to enhance apoptosis. This will be discussed in more detail in Section 1.6.5.

1.6.2 Adhesion molecules and the development of Atherosclerosis

Migration of monocytes into the sub-endothelial space is essential for the initial stages of the atherosclerotic lesion development as they differentiate into macrophages and take up oxidized LDL. This leads to the formation of foam cells. As outlined previously, migration of monocytes is dependent on the expression of a number of different cell adhesion molecules (CAM) on the endothelial cell surface. There are two main families expressed in endothelial cells including; P and E selectins and the member of immunoglobulin superfamily, ICAM and VCAM. Regulation of CAM expression is crucial and all three types of CAM acting cooperatively in the process of monocyte transmigration into sub-endothelial space (Chia, 1998). Selectins are the first class CAM which regulates the initial weak attachment of monocytes to the endothelium by a process called "rolling", which is a low-affinity binding of selectin to carbohydrate molecules on the monocyte (Dong et al., 1998). E-selectin expression is observed in the atherosclerotic region of blood vessels but not in normal segments (O'Brien et al., 1996), therefore it is more prevalent in atherosclerotic plaque. Additionally, there is an increase in arterial segments showing mononuclear leukocyte accumulation where E-selectin is also expressed. This suggests E-selectin plays a role in monocyte migration in lesion development (O'Brien et al., 1996). ICAM-1 and VCAM-1 are identified as a second class of CAM. These molecules mediate the formation of firm endothelium-monocyte adhesion via interacting with integrins which are expressed on monocytes (Elices et al., 1990, Marlin and Springer, 1987). Moreover, there is evidence which links ICAM-1 deficiency to reduced atherosclerotic lesions in ApoE/ICAM-1 double-knockout mice (Bourdillon et al., 2000), suggesting that both ICAM-1 and VCAM-1 play crucial roles in monocyte recruitment and initial lesion development. The third class of CAM, the integrins, are expressed on leukocytes. Integrins act as ligands for ICAM-1 and VCAM-1; β2 integrin, LFA-1 and Mac-1 bind to ICAM-1 while VLA-4 is specific for VCAM-1 (Elices et al., 1990, Marlin and Springer, 1987).

In addition to expression of adhesion molecules, a number of other factors are required for the migration of monocytes into the sub-endothelial space. Following firm adhesion,

monocytes transmigrate through the endothelial layer into the tunica intima. This process requires chemokines such as monocyte chemoattractant protein-1 (MCP-1) (Harrington, 2000). MCP-1 is classified as the C-C subfamily of chemokines and acts as a potent monocyte chemoattractant when expressed on vascular endothelial cells. MCP-1 was shown to be expressed on atheromatous lesions (Tanaka et al., 2003), and regulates monocyte chemotaxis, which draws firmly attached monocytes into the arterial intima through interaction with its ligand on monocytes, chemokine receptor-2 (CCR-2) (Hemmerich et al., 1999, Salcedo et al., 2000).

After monocytes migrate into sub-endothelial spaces, they differentiate into macrophages in response to monocyte colony stimulating factor (M-CSF) (Krishnaswamy et al., 1999, Sakai et al., 2000). They express receptors that specifically recognize oxidized LDL, including scavenger receptor A (de Villiers and Smart, 1999) and CD36 (Nicholson et al., 2000, Silverstein and Febbraio, 2000) and produce pro-inflammatory cytokines such as TNF-α and IL-1. Inflammation gives rise to lipid peroxidation, and lipid peroxidation in turn results in more inflammation, triggering the first step in the development of an atherosclerotic lesion (Kutuk and Basaga, 2003). In addition to a role for adhesion molecules, evidence implicates an involvement of a family of proteolytic protein known as the matrix metallo-proteinases (MMPs). This comprises at least 12 MMP isoforms and endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). A recent study shows that inhibition of MMP-12 prevents atherosclerotic plaque development and increases stability in $ApoE^{-/-}$ mice (Johnson et al., 2011). As MMPs play a significant role in the degradation of proteins within the extracellular matrix (ECM) (Ra and Parks, 2007), overexpression of MMPs allow vascular smooth muscle cells to transmigrate into the intima during atherosclerosis. Moreover, there is a study which demonstrates that MMP-2 is responsible for vascular smooth muscle cell proliferation in response to oxidized LDL (Auge et al., 2004) which is strongly associated with atherosclerotic development. Figure 1.5 shows the cellular mechanisms underpinning the development of atherosclerosis.

1.6.3 NFκB and atherosclerosis

Given that inflammation is a key aspect of atherosclerosis development, it is perhaps not surprising that a considerable body of evidence supports a role for NFκB in this process. Activation of NFκB is associated with the development of early lesions (Kutuk and Basaga,

2003). In addition, using immunofluorescence and immunohistochemistry, early studies showed activated NFκB in macrophages, endothelial cells and smooth muscle cells located in the media and intima of thickened fibrous atherosclerotic regions of the vessel wall. By contrast, little or no activation of NFκB was detected in otherwise healthy parts of the arteries (Brand et al., 1997a, Brand et al., 1996, Brand et al., 1997b). Later, another research team demonstrated activation of NFκB in the intimal layers of human coronary artery plaques (Wilson et al., 2002).

There is accumulating evidence to suggest that inhibiting NFκB can attenuate atherosclerosis. In apoE/LDLR double knockout mice, lesion development was reduced by an NFκB inhibitor, PDTC (Jawien et al., 2005). A similar study also showed that ablation of IKKγ or expression of a dominant-negative IκB-α, specifically in endothelial cells, resulted in strong reduction of atherosclerotic plaque formation in $ApoE^{-/-}$ mice fed with a cholesterol rich diet (Gareus et al., 2008). This effect was associated with reduced adhesion molecule induction in endothelial cells and impaired macrophage recruitment to the atherosclerotic plaque (Gareus et al., 2008). This data suggests that inhibiting the NFκB pathway may attenuate atherosclerosis, however, the situation is made more complicated by the fact that inhibiting NFκB in certain cell types may also aggravate atherosclerosis. One knockout study has demonstrated that inhibition of the NFκB pathway by removal of IKKβ in macrophages lead to an increase in lesion size. Moreover, the condition of the lesion became more severe due to increased necrosis and more macrophages in the early lesions (Kanters et al., 2003). Therefore, activating the NFκB pathway may lead to expression of certain genes expected to ameliorate atherosclerosis such as IL-10 which mediates the condition. Therefore inhibiting NFκB may attenuate atherosclerosis if targeted specifically in certain cell types and to specific gene clusters.

An overwhelming number of cellular studies performed *in vitro* correlate with effect observed *in vivo*. In endothelial and vascular smooth cells and macrophages, NFκB is strongly linked with ICAM-1 and VCAM-1 expression. At a cellular level, these adhesion molecules are well recognized to be expressed on vascular endothelial cells following NFκB activation (Bunting et al., 2007, Denk et al., 2001, Huang et al., 2007, Iademarco et al., 1992, MacKenzie et al., 2007, van de Stolpe et al., 1994). Other molecules intimate to cellular migration such as MCP-1 are also regulated by canonical NFκB. Furthermore, NFκB regulates the expression of many cytokines (TNF-α and IL-1β) and chemokines involved in

the initiation of atherosclerotic lesions including monocyte recruitment into the vessel wall (Cominacini et al., 2000, Rahman et al., 1998, Zhu et al., 1999).

1.6.4 Oxidized LDL and NFκB

Atherosclerotic lesions are also regulated by further oxidation of lipids and further secretion of inflammatory mediators by macrophages (Saxena and Goldberg, 1994). For initiation of atherosclerotic lesions development, lipid deposition is necessary and is preceded by initial LDL oxidation. Several studies have demonstrated that oxidized LDL strongly activates NFκB pathway in monocytes resulting in an enhance release of pro-inflammatory cytokines including TNF-α, IL-1β, IL-8 and IL-12 (Brand et al., 1997a, Janabi et al., 2000, Mytar et al., 2004). The oxidized LDL has prominent pro-inflammatory properties (Reddy et al., 2002) and might act as an early mediators of chronic inflammatory reactions through activation of NFκB. This results in induction of the expression of cytokines and cell adhesion molecules which are important factors for the migration of monocytes into the sub-endothelial space (Cushing et al., 1990, Rajavashisth et al., 1995). In addition, Parhami and co-workers also demonstrated that minimally oxidized LDL, activates NFκB in endothelial cells (Parhami et al., 1993).

Oxidized LDLs are thought to exert biphasic effects on NFκB activity. Low concentrations of oxidized LDL promote inflammatory effects by up-regulating inflammatory gene expression via NFκB activation, however, high levels of oxidized LDL mediates immunosuppressive effects by inhibiting NFκB activation in response to inflammatory agents such as LPS (Robbesyn et al., 2004). Indeed, Wang and co-workers have shown that incubating HUVECs with LPS causes inflammatory gene expression via TLR4/NFκB activation and induces the expression of TLR4, LOX-1, ICAM-I, and E-selectin, and increases monocyte adhesion to endothelium (Wang et al., 2005). These data, together with other works which showed that increasing NFκB can prevent oxidised LDL induced apoptosis (Nishio et al., 2000), suggests that NFκB has the potential to act as a double-edged sword in relation to oxidized LDL function and atherosclerotic development.

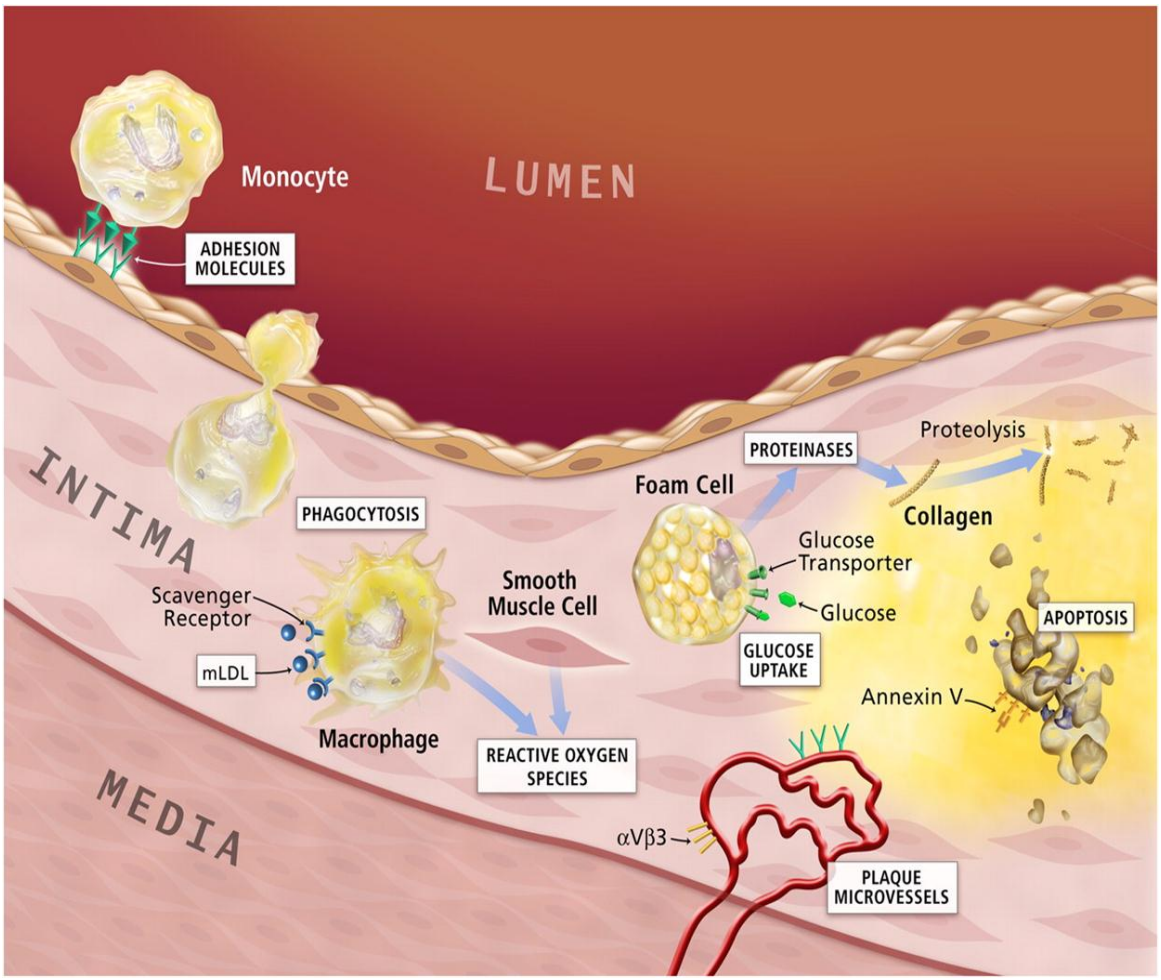


Figure 1.5 Cellular mechanism regulating atherosclerosis. The diagram shows the expression of adhesion molecules facilitate the transmigration of monocyte into subendothelial layer. Adopted from (Libby et al., 2010a)

1.6.5 Atherosclerosis development and endothelial dysfunction and apoptosis

A key cellular event in endothelial dysfunction and implicated in atherosclerosis is apoptosis. Apoptosis is controlled programmed cell death which is regulated by a number of key signaling events. Many classic atherosclerotic risk factors such as oxidized LDL (Dimmeler et al., 1997), increased oxidative stress (Lizard et al., 1997) and angiotensin II (Dimmeler et al., 1997) cause endothelial cell apoptosis. Furthermore, the inflammatory environment within the atherosclerotic plaque leads to expression of iNOS which gives rise to a high level of NO and oxygen radicals (Buttery et al., 1996, Luoma et al., 1998, Wilcox et al., 1997). In this situation NO itself could induce apoptotic cell death and destabilise the atherosclerotic plaque (Beckmann et al., 1994, Foresti et al., 1999). Moreover, high concentration of pro-inflammatory cytokines such as TNF-α and IL-1β found in the atherosclerotic lesion may also contribute to endothelial apoptosis (Frostegard et al., 1999).

Despite these findings, the involvement of NFκB in regulating apoptosis in the context of atherosclerosis is problematic. Whilst inhibiting the expression of inflammatory proteins such as ICAM-1 (van de Stolpe et al., 1994) and VCAM-1(Iademarco et al., 1992) through blocking the canonical pathway may be beneficial, this is detrimental to endothelial cell survival. NFκB regulates pro-survival proteins such as bcl-2 (Catz and Johnson, 2001) and cFLIP (Kreuz et al., 2001) and also anti-apoptotic proteins such as X-linked inhibitor of apoptosis protein (XIAP) (Turner et al., 2007) and porcine inhibitor of apoptotic protein (PIAPs) (Stehlik et al., 1998a). Moreover, VEGF expression is up-regulated by TNF-α induced activation of NFκB pathway which aids endothelial cell survival. However VEGF is also linked to increased adhesion molecule expression which would indicate a role in atherosclerotic development. Furthermore NFκB can itself contribute to endothelial cell apoptosis through the induction of iNOS in macrophages and enhanced formation of peroxynitrite (Cromheeke et al., 1999). Bombeli and co-workers have shown that apoptotic HUVECs become pro-coagulant via increased expression of phosphatidylserine (PS) and loss of anti-coagulant membrane components (Bombeli et al., 1997), suggesting that apoptosis can accelerate the development of atherosclerosis.

These studies are relevant when considering the therapeutic targeting of the canonical NFκB pathway. Blocking p65 NFκB translocation or direct pharmacological inhibition of IKKβ will often result in endothelial cell apoptosis which will be detrimental to vessel integrity.

Therefore approaches which involve inhibiting the expression of pro-inflammatory molecules such as ICAM-1 and VCAM-1 whilst leaving pro-survival genes unaffected would be desirable. Inhibition of IKKα may therefore be an attractive approach however, it is not supported as yet by studies demonstrating a role for IKKα in the development of atherosclerosis.

1.7 Angiogenesis

1.7.1 Introduction to Angiogenesis

Angiogenesis is the process of vascular growth by sprouting of pre-existing vessels. It is subject to a complex control system with pro-angiogenic and anti-angiogenic factors. In adults, angiogenesis is strictly regulated. The formation of new blood vessels occurs during wound healing, organ regeneration, and in the female reproductive system during ovulation, menstruation, and the formation of the placenta in normal circumstances. Uncontrolled angiogenesis is strongly related to several pathological processes such as cancer development, rheumatoid arthritis, diabetic retinopathy and psoriasis (Hoeben et al., 2004, Munoz-Chapuli et al., 2004). Angiogenesis involves a large number of endothelial cell processes, therefore many extracellular signals are involved. A growth factor central to angiogenesis is VEGF. VEGF virtually regulates almost every aspect of angiogenesis involving endothelial cells including; proliferation (Kanno et al., 2000, Wu et al., 2000), survival (Nor et al., 1999), migration (Favot et al., 2003), extracellular matrix degradation (Funahashi et al., 2011), differentiation and morphogenesis (Stratman et al., 2011). Other growth factors can also play a role, for instance, activation of FGF receptor signalling mechanisms can trigger endothelial cell proliferation (Cabrita and Christofori, 2003, Eliceiri et al., 1999, Hanafusa et al., 2002). Angiopoietin receptors, also called Tie receptors, are also important in regulating cell migration (Carlson et al., 2001). Activation of PI3K pathways through these receptors can also up-regulate the pro-survival genes such as Bcl-2 to enhance endothelial cell integrity (Catz and Johnson, 2001).

1.7.2 NFκB and Angiogenesis

The role of NFκB in regulation of angiogenesis remains unclear. On the one hand, NFκB regulates the expression of proteins which may be involved in the angiogenic process such as MMPs 2, 3 and 9 (Agarwal et al., 2005, Mountain et al., 2007, Popov et al., 2006), VEGF

(Grosjean et al., 2006, Ko et al., 2006), IL-6, MCP-1 (Denk et al., 2001) and IL-8 (Agarwal et al., 2005). On the other hand, NFκB can also enhance the expression of anti-angiogenic factors such as Plasminogen Activator Inhibitor 1 (PAI-1) (Hou et al., 2004), thrombospondin-1 (TSP-1) (Yang et al., 2004) and Vascular Endothelial Growth Inhibitor (VEGI) (Xiao et al., 2005). A number of these genes can be differentially regulated by the canonical and non-canonical NFκB pathway and this is discussed below. Therefore understanding the role of each of the NFκB pathways could be beneficial for the treatment of cardiovascular diseases.

1.7.3 The Canonical NFκB Pathway and Angiogenesis

As mentioned previously, the IKKβ/p65 axis regulates expression of a large number of proteins which have been directly or indirectly linked to angiogenesis through effects on endothelial cell proliferation and survival. This includes not only XIAP and c-IAP (Chu et al., 1997, Stehlik et al., 1998a) but other proliferative genes such as Ephrin-A1 (Deregowski et al., 2002), IL-6 (Plumpe et al., 2000) and cyclin D1 (Hinz et al., 1999). Inhibition of p65 also results in a decrease in the expression of angiopoietin-1 (Scott et al., 2005) and 2 (Kim et al., 2000) both of which induced endothelial cell proliferation and enhance angiogenesis.

Endothelial cell migration which is intimate to angiogenesis is also coordinated by p65-dependent regulation of both MMPs expression and the corresponding inhibitors of MMPs such as TIMP-1 (Wilczynska et al., 2006). Furthermore, Swiatkowska and co-workers found TNF-α induced the expression of PAI-1, a protein that negatively regulates plasmin formation and as a consequence angiogenesis via activation of the canonical NFκB signalling pathway (Swiatkowska et al., 2005). Activation of p65 by TNF-α can also inhibit tPA expression to further reduce plasmin levels (Ulfhammer et al., 2006). In contrast, there is some evidence to suggest that NFκB can inhibit angiogenesis. VEGI is up-regulated by p65 (Xiao et al., 2005), as is thrombospondin-1 (TPS-1), an anti-angiogenic agent which is responsible for inhibiting endothelial cell proliferation and migration, it has been demonstrated that p65 binds to the promoter sites of these genes to induce transcription activity (Yang et al., 2004). These results suggest that p65 may also negatively regulate extracellular matrix degradation and hence inhibit angiogenesis.

In contrast to a large number of cellular studies, there is much less evidence demonstrating a role for the canonical pathway in angiogenesis *in vivo.* A study using Tie2 promoter/enhancer–IκBα$^{S32A/S36A}$ transgenic mice which carried mutation in the critical serines at 32 and 36 in IκB-α resulting in p65 inactivation revealed a striking increase in tumour vascularisation in transgenic mice compared to control (Kisseleva et al., 2006).

1.7.4 The Non-Canonical Pathway and Angiogenesis

Whilst the number of studies examining the role of the non-canonical pathway and in particular IKKα, in the regulation of angiogenesis are limited, there is nevertheless some evidence to support this hypothesis. In endothelial cells, lymphotoxin a known activator of IKKα was found to induce expression of pro-angiogenic factors, for instance, interleukin-8 (IL-8), chemokine (C-X-C) motif ligand 12 (CXCL12) (Liekens et al., 2010) and also increase VEGF synthesis and tubule formation (Liang et al., 2007). Furthermore, DeBusk and co-workers demonstrated that over-expression of IKKα in endothelial cells increased cell migration and also the number of vascular branch points (DeBusk et al., 2008). In addition, IKKα is also responsible for regulation of MMP-2, MMP-9, Urokinase plasminogen activator (uPA) and VEGF (Agarwal et al., 2005). These genes are along with IL-8, also responsible for cell migration and extracellular matrix degradation, so activation of the non-canonical pathway may be important in this process and as a result, angiogenesis. Overall, it would seem that the canonical NFκB pathway plays a role in angiogenesis through regulation of endothelial cell proliferation and survival, whilst the non-canonical pathway is involved predominantly in cellular migration and extracellular matrix degradation. This possibility, whilst requiring more studies suggests that these two processes could be targeted differentially using selective inhibitors. Figure 1.6 highlights the individual roles of both canonical and non-canonical NFκB pathways in the regulation of angiogenesis.

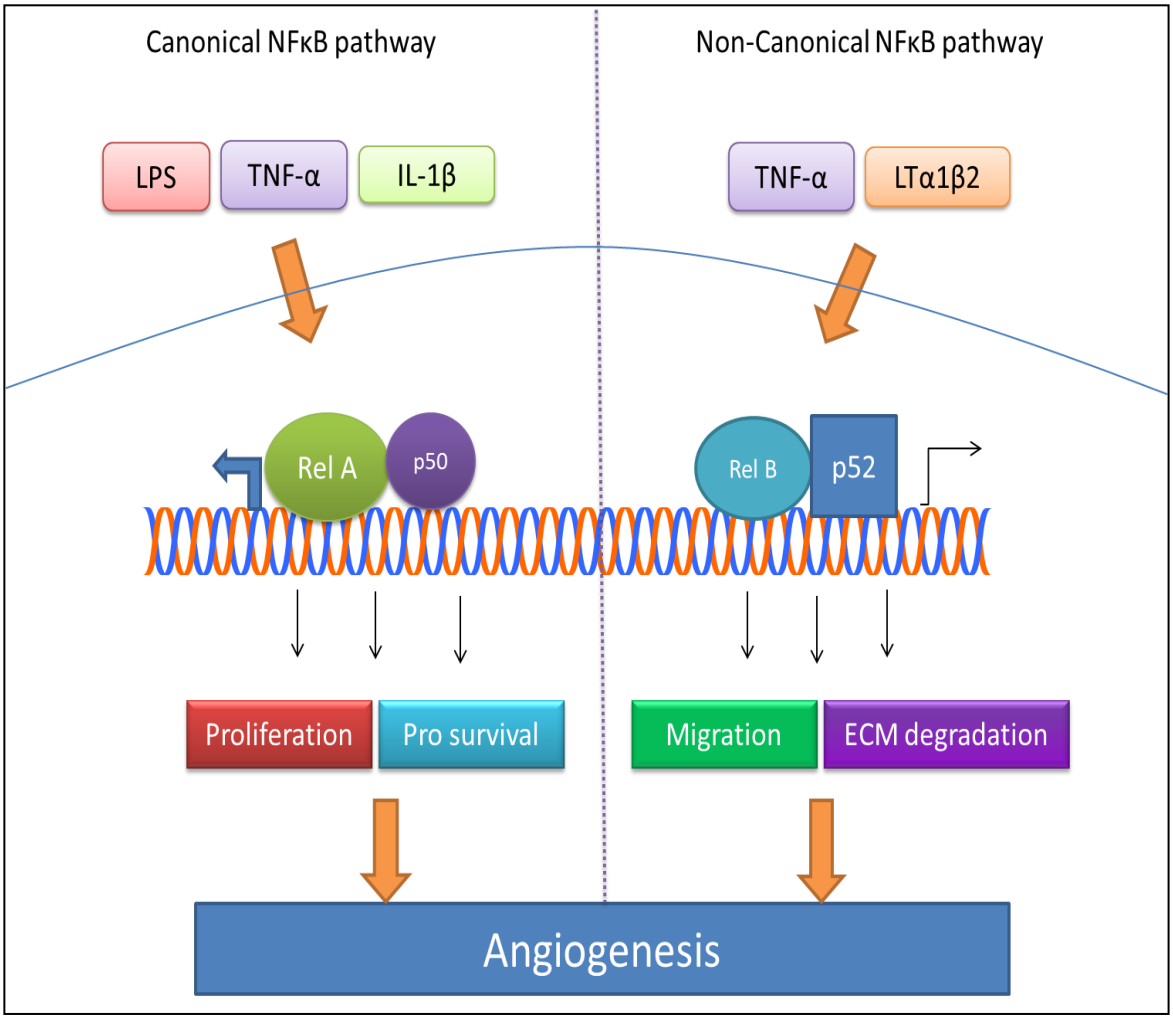


Figure 1.6 The role of the NFκB pathways in the regulation of angiogenesis. The canonical NFκB pathway and non-canonical NFκB pathway play different role in the regulation of angiogenesis process. The canonical NFκB pathway is responsible for cell proliferation and survival, whilst the non-canonical NFκB is responsible for cell migration and extracellular matrix degradation.

1.8 The development and use of IKKα inhibitors

As described above, there is considerable evidence which supports a role for NFκB in processes such as angiogenesis and chronic inflammatory diseases including rheumatoid arthritis and atherosclerosis. Therefore inhibition of NFκB may be an important therapeutic strategy in the treatment of those diseases. Several NFκB inhibitors have been developed for experimental and clinical use including PDTC (Liu et al., 1999), disulfiram (Wang et al., 2003), BAY 117082, MG132 (Dimmeler et al., 1999) and selective p65 NFκB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ). However these agents have a wide range of actions within the pathway. As IKKs play a key role in the regulation of the NFκB pathway inhibiting specifically the IKKs would be beneficial in the treatment of a number of diseases. With the discovery of IKKs, a considerable amount of effort has been put into the development of small molecules IKK inhibitors. Currently the available compounds are directed to and selective for IKKβ. The majority of these inhibitors are ATP-competitive molecules or alternatively possessing allosteric actions to limit IKK activities (Gamble et al., 2012). In addition, there are several natural products which can weakly inhibit IKK including curcumin, (-)-epigallocatechin-3-gallate, parthenolide and quercetin (Muller and Siebenlist, 2003), but these are not supplied appropriate lead molecules.

Castro and co-workers discovered the β-carboline compounds such as PS-1145 inhibited the phosphorylation of IκB-α, NFκB activation and TNF-α expression by inhibiting IKK (Castro et al., 2003). Other classes of IKKβ inhibitors including N-(6-chloro-9H-β-carbolin-8-yl) nicotinamide (PS-1145) and N-(6-chloro-7-methoxy-9H-β-carbolin-8-yl)-2-methyl-nicotinamide (ML120B) also inhibited the expression of NFκB (p65/p50) dependent genes such as ICAM-1, IL-6, IL-8 GM-CSF, RANTES, MCP-1, GROα, NAP-2 and ENA-78. Furthermore, in vivo, these inhibitors were found to be as effective as synthetic steroids which inhibited NFκB indirectly for example dexamethasone. There is also therapeutic potential for these inhibitors in the treatment of inflammatory airway diseases, particularly in chronic obstructive pulmonary disease (COPD) or severe asthma (Catley et al., 2005). In relation to cancer, Gasparian and co-workers showed proteasome inhibitors such as epoxomicin and MG132 attenuated NFκB activity much more effectively than the IKK inhibitors BAY 11-7082 and PS-1145 in androgen-sensitive human prostate adenocarcinoma cells (LNCaP) (Gasparian et al., 2009). Overall evidence in this chapter has identified the potential of IKKα as a distinctive regulatory kinase in both canonical and non-canonical

NFκB pathways and therefore may represent a novel drug target. Consequently development of selective IKKα inhibitors would be a potential break-through in terms of therapeutic targeting of disease.

1.9 Aims and Objectives

Inhibition of IKKβ is a modern therapeutic approach in treatment of cancers and several inflammatory diseases. However, there is a limitation in the use of IKKβ inhibitors for the treatment of atherosclerosis. The beneficial effect of an IKKβ inhibitor would be to block the expression of inflammatory mediators, however this may also lead to a decrease in anti-apoptotic protein expression. In the context of blood vessel integrity, endothelial cells would undergo apoptosis. This chapter has reviewed evidence supporting a role for IKKα in endothelial function. Although overall this role remains uncertain, there may be therapeutic potential if IKKα is predominant in the regulation of the inflammatory mediators rather than the cell death programme. In this thesis, the following aims will be addressed in two main result chapters.

1. Characterisation of the role of IKKα in regulation of NFκB pathways in HUVECs by:
i) Determining the role of IKKα in regulation of canonical NFκB pathway by overexpression of DN-IKKα.
ii) Assessing the effect of IKKα in the regulation of the adhesion molecule expression following overexpression of DN-IKKα or knockdown of IKKα (siRNA) in HUVECs.
iii) Examining the function of IKKα in regulation of the non-canonical NFκB pathway by overexpression of DN-IKKα or knockdown of IKKα (siRNA) in HUVECs.

2. Characterisation of the pharmacological effects of putative selective IKKα inhibitors by:
i) Examining the selectivity of IKKα inhibitors within the non-canonical NFκB pathway.
ii) Assessing the inhibitory effect of p100 processing with the IKKα inhibitors.
iii) Assessing the inhibitory effect of adhesion molecules expression with the IKKα inhibitors.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General Reagents

All materials used were of highest commercial purity available and were supplied by Sigma Aldrich Chemical Company Ltd. (Poole, Dorrset UK) or other reputable companies unless otherwise stated.

Bio-Rad Laboratories (Hertfordshire, UK)

Bio-Rad DC™ Protein Assay Dye Reagent Concentrate

Pre-stained SDS-Page molecular weight markers.

Boehringer Mannheim (East Sussex, UK)

Bovine Serum Albumin (BSA , Fraction V)

Fisher Chemical UK Limited (Leicestershire, UK)

Bovine Serum Albumin (BSA , Fraction V)

Insight Biotechnology Limited (Wembley, UK)

Recombinant Human Interleukin-1 beta (IL-1β)

Recombinant Human Tumour Necrosis Factor-alpha (TNF-α)

GE Healthcare (Buckinghamshire, UK)

Amersham Hybond ECL Nitrocellulose Membrane

Carl Roth GmbH + CO. KG (Karlsruhe, Germany)

Rotiphorese® Gel 30 (37.5:1) acrylamide

Whatmann (Kent, UK)

Nitrocellulose Membrane, 3MM blotting paper

GIBCO BRL (Paisley, UK)

Foetal Calf Serum (FCS), L-glutamine, Minimal Essential Medium (x10), Non-essential amino acids, Penicillin/Streptomycin, Sodium Bicarbonate.

Corning B.V (Buckinghamshire, UK)

All tissue culture flasks, plates, dishes and graduated pipettes

Sarstedt AG & Co LTD (Leicester, UK)

Serological pipette 5ml

Serological pipette 10ml

Thermo Fisher Scientific Inc (Surrey,UK)

Multidish 6 wells

Multidish 12 wells

Roche Diagnostics Ltd (Burgess Hill, UK)

FuGENE® HD Transfection Reagent

Dithiothreitol (DTT)

Lonza (Slough, UK)

Endothelial Cell Basal Medium-2 (EBM-2)

Endothelial Growth Media (EGM^{TM}-2) SingleQuots

YFP-tag IKKα

YFP-tag NFκB p65

2.1.2 Adenoviruses

Clontech Laboratories Inc (Mountain view, CA, USA)

Adeno-X virus purification kit

Vector Biolabs (Philadelphia, USA)

NFκB-Luciferase Human Adenovirus type 5 (Adv.NFκB-Luc)

Invitrogen Corp (California, USA)

shRNA IKKα

shRNA IKKβ

The following adenoviruses were kindly provided by Dr D.Goeddel (Tularik Inc., CA, USA)

Adv. DN-IKKα

Adv. DN-IKKβ

2.1.3 SU compounds (University of Strathclyde, Glasgow, UK)

SU937

SU1007

SU1010

SU1020

SU1053

2.1.4 Microscopy

Merck-Calbiochem (Nottingham, UK)

Mowiol

Molecular Devices Corp (Downingtown, PA, USA)

MetaMorph Imaging Series software (version 7.0 or 7.6.4)

Nikon Instruments (New York, USA)

Nikon TE-300 Epifluorescence microscope

Nikon Eclipse TE2000-E inverted Epifluorescence microscope

40x or 100x oil-immersion Plan Fluor Objective Lens, NA=1.3

VWR International Ltd (Leicestershire, UK)

No. 0, (0.09-0.13 mm thick), circular glass 13 or 22 mm diameter coverslips.

0.8-1.0mm thick glass microscopy slides

2.1.5 Antibodies

Santa Cruz Biotechnology Inc (California, USA)

Rabbit monoclonal anti-IκBα (C-21)

Rabbit polyclonal IKKα/β (H-470)

Rabbit polyclonal NFκB p65 (C-20)

Mouse monoclonal Actin (C-2)

Goat polyclonal IKKβ (C-20)

Cell signaling Technology Inc (Hertfordshire, UK)

Rabbit polyclonal p-p65 (Ser^{536})

Rabbit polyclonal Histone H_3

Calbiochem (Nottingham, UK)

Mouse monoclonal anti-IKKα (14A231)

R&D Systems Europe Ltd (Oxon, UK)

Biotinylated Anti-human VCAM-1 (CD106) (BAF809)

Biotinylated Anti-human ICAM-1 (BAF720)

Trevigen Inc (MD, USA)

TACS® Streptavidin-Horseradish Peroxidase Conjugate (Strep-HRP)

Jackson ImmunoResearch Laboratories Inc (PA, USA)

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (111-035-144)

Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG

Texas Red dye-conjugated AffiniPure donkey anti-goat IgG (705-076-147)

Millipore (U.K.) Limited (Watford, UK)

Anti-NFκB p52 - 32534

2.2 CELL CULTURE

All cell culture work was performed in a class II cell culture hood under aseptic conditions. All the cells were grown in 75cm^2 flasks unless otherwise stated.

2.2.1 Human Umbilical Vein Endothelial Cells (HUVECs)

Cryopreserved Primary HUVECs (≥ 500,000 cells/vial) were purchase from Cascade Biologics. HUVECs were maintained in Endothelial Basal Media (EBM-2) supplemented with EGM-2 single Quots (2% Foetal bovine serum, 0.2ml Hydrocortisone, 2ml hFGFB, 0.5ml VEGF, 0.5ml R3-Insulin like Growth Factor-1, 0.5ml Human Epidermal Growth Factor (hEGF), 0.5ml Gentamicin, Amphotecerin-B (GA-1000), 0.5ml heparin and Ascorbic acid, 0.5ml). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Medium was changed every 2 days thereafter until cells became confluent.

2.2.2 Subculturing HUVECs by Trypsinisation

Cells were subcultured upon reaching approximately 90% confluency. The media was removed and the cells washed twice with a sterile solution of 0.5% (w/v) trypsin, 0.2% (w/v) EDTA in phosphate buffered saline (PBS). The solution was then removed and the flask placed in an incubator at 37°C, 5% of CO_2 and 95% air, for 2-5 min until cells began to round up, indicating that they had begun to detach from the flask. The flask was then gently tapped, to facilitate the cells to completely detach from the surface and then wash in EBM-2. Cells were then diluted 3 times with additional EBM-2 and transferred to fresh flasks (75 cm^2) or seeded into plates (12 or 6 wells). Cells were maintained at 37°C in an incubator and the media replaced every 2 days until they reach 90% confluency and ready for stimulation). Cells were used for experimentation between passages 2 to 6 and were stimulated with different reagents in full media.

2.2.3 Human Embryonic Kidney (HEK293)

Low passage human embryonic kidney (HEK) 293 cells were maintained in Minimal Essential (MEM) with Eagle's salts supplemented with 10% (v/v) foetal calf serum (FCS), penicillin (250 units/ml), streptomycin (100µg/ml), L-glutamine (27mg/ml), 1x (v/v) non-essential amino acid and 0.375% (v/v) sodium bicarbonate (all GIBCO®, Invitrogen Ltd). Cells were then incubated at 37°C in a humidified atmosphere with 5% CO2 with media replaced every 2 days. At 90% confluence, HEK 293 cells were passaged using 1 x sodium sodium citrate (SSC) solution (0.00878kg NaCl (1.5mM) and 0.00441kg (0.54mM) sodium acetate dissolved in 100ml of distilled water, then adjusted to pH 7.0). Cells were used for experimentation between passage 30 to 40.

2.3 WESTERN BLOT ANALYSIS

2.3.1 Preparation of Whole Cell Extracts

Cells were exposed to appropriate agonists or vehicle for the relevant period of time and they were then placed on ice to stop the reaction (protein degradation/ dephosphorylation). Cells were immediately washed twice with ice cold PBS before 150µl of pre-heated laemmli's sample buffer (63mM Tris-HCl (pH6.8), 2mM $Na_4P_2O_7$, 5mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50mM DTT, 0.007% (w/v) bromophenol blue) was added. The cells were then scraped and the chromosomal DNA sheared by repeatedly pushing through a 21 gauge needle. The cells were then transferred to Eppendorf tubes and boiled for 5min to denature the proteins in the samples, before keeping at -20°C or -80°C until use.

2.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel kit apparatus was first cleaned in 70% ethanol before assembly, then distilled water was added overnight to check the glass plates were flush and not leaking. Resolving gels were prepared containing an appropriate amount (7.5 (w/v) %, 8.5 (w/v) %, 10 (w/v) % 15 (w/v) %) acrylamide (Rotiphorese® Gel 30 (37.5:1), 0.375M Tris base (pH8.8), 0.1% (w/v), SDS and 10% (w/v) Ammonium persulfate (APS)). Polymerisation was initiated by the addition of 0.05% (v/v) N, N, N', N' tetramethylethylenediamine (TEMED). The solution was poured between 2 glass plates assembled in a vertical slab configuration according to the manufactures instruction (Bio-Rad) and overlaid with 180µl of 0.1% (w/v) SDS. Following gel polymerization the layer of 0.1% (w/v) SDS was removed and a stacking gel containing 10% (v/v) acrylamide: Rotiphorese® Gel 30 (37.5:1), 0.125M Tris base (pH6.8), 0.1% (w/v) SDS and 10% (w/v) Ammonium persulfate (APS) and 0.05% (v/v) TEMED) was poured directly on top of the resolving gel, and a teflon comb was immediately inserted into the stacking gel solution. After polymerization was complete, the comb was removed and the polyacrylamide gels were assembled in a Bio-Rad Mini-PROTEAN 3™ electrophoresis tank, with both reservoirs filled with electrophoresis buffer (25mM Tris, 129mM glycine, 0.1% (w/v) SDS). Aliquots of samples (20-30µg/ml) were then loaded into the wells using a microsyringe. A prestained SDS-PAGE molecular weight marker of known molecular weights was run concurrently in order to identify the polypeptide of interest. Samples were electrophoresed at a constant voltage of 120V, until the bromophoenol dye had reached the bottom of the gel.

2.3.3 Electrophoretic Transfer of Proteins to Nitrocellular Membrane

The proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by electrophoretic blotting following a standard protocol (Towbin et al., 1979). The gel was pressed firmly against a nitrocellulose sheet and assembled in a transfer cassette sandwiched between 2 filter papers and two sponge pads. The cassette was immersed in transferred buffer (25M Tris, 19mM glycine, 20% (v/v) methanol) in a Bio-Rad Mini-PROTEAN 3^{TM} electrophoresis tank and a constant current of 300mA was applied for 1h and 45min, whilst the tank was cooled by inclusion of an ice reservoir. The presence of SDS in the resolving gel confers a negative charge on the protein so the cassette was oriented with the nitrocellulose towards the anode.

2.3.4 Immunological Detection of Protein

Following transfer of the proteins from the gel to the nitrocellulose membrane, the membrane was removed and any remaining protein blocked by incubation in a solution of 2% (w/v) BSA in NaTT buffer (150mM NaCl, 20mM Tris (pH 7.4), 0.2% (v/v) Tween -20) for 90min with gentle agitation on a platform shaker. The blocking buffer was then removed and membranes incubated overnight with antiserum specific to the target protein appropriately diluted in NaTT buffer containing 0.2% (w/v) BSA. On the following day, membranes were washed in NaTT every 15min for 90min with gentle agitation. The membranes were then incubated for a further 90min at room temperature with secondary horseradish peroxidase-conjucated IgG directed against the first immunoglobulin diluted to approximately 1:10000 in NaTT buffer containing 0.2% (w/v) BSA. After 6 washes in NaTT for 90min as described before, immunnoreactive protein bands were detected by incubation in enhanced chemiluminescence (ECL) reagent for 2min with agitation. The membranes were then mounted onto an exposure cassette and covered with cling film, then exposed to X-ray film (B Plus – Full Blue) for the required time under darkroom conditions and developed using X-OMAT machine (KODAK M35-M X-OMAT processor).

2.3.5 Nitrocellulose membrane stripping and reprobing

Nitrocellulose membranes processed by Western Blotting were reprobed for the subsequent detection of other cellulose proteins. This involved stripping the membrane of any previous antibody using a stripping buffer (0.05M Tris-HCl, 2% SDS, and 0.1M of β-

mercaptoethanol). The membrane was incubated in 15ml of stripping buffer for 60min at 70°C in an incubator/shaker (Stuart Science Equipment). The stripping buffer was then discarded in a fume hood sink and the membrane washed 3 times with NaTT buffer at 15min intervals to remove residual stripping buffer. After the final NaTT wash, membranes were then incubated overnight with primary antibody prepared in 0.2% BSA (w/v) in NaTT buffer. At this stage, the blots were ready for the immunological detection protocol (section 2.34).

2.4 Preparation of nuclear extract

Cells were grown on 10cm plates until 90% confluency and then exposed to vehicle or agonist for indicated time period. After washing with 1ml of ice cold PBS, cells were scraped into 0.5ml PBS and transferred to Eppendorf tubes. Cells were then harvested by centrifugation at 13000 rpm for 1min. The pellet was resuspended in 400µl of buffer 1 (10mM Hepes pH 7.9, containing 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin and 10µg/ml pepstatin) and incubated on ice for 15min. 25µl of 10% (w/v) NF-40 was added into the samples and vortexed at full speed for 10s. The nuclear fraction was then separated by centrifugation at 13000 rpm for 1min and supernatant were removed and pellet were resuspended in 50µl of buffer 2 (20mM Hepes, (pH 7.9), 25% (v/v) glycerol, 0.4M NaCl, 1mM EGTA, 1mM DTT, 0.5mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin and 10µg/ml pepstatin) and agitated at 4°C for 15min. Extracts were then sonicated in ice bath for 2 x 30s. Nuclear proteins were then recovered by centrifugation and the soluble nuclear extract collected and stored at -80°C until use.

2.4.1 Determination of Protein Concentration in the Nuclear Extracts

Quantification of protein concentration was determined using the Bio-Rad DC™ Protein Assay Dye Reagent Concentrate Farbstoff-Konzentrat on the Bradford assay method. A standard curve was performed using various concentrations (2-20µg) of BSA. Appropriate dilutions of the standards and samples were made in sterile dH_2O (790-795µl) mixing with 200µl of dye agent and left for 15min at room temperature for the reaction. Samples were transferred into a cuvette and the colour development was quantified at 595nm by Ultrospec®2000 UV/visible spectrophotometer. The protein concentration of each sample was calculated from the standard curve.

2.5 Luciferase reporter activity assay

Adenovirus encoding luciferase gene containing NFκB binding sites was purchased from (Vector Biolabs Inc). HUVECs were grown to confluency in 12 well plates then after infected with Adv. NFκB luc for 40h prior stimulation with TNFα at the indicated time points. Cells were exposed to agonists for 8h, followed by washing twice with ice cold PBS and 100µl of lysis buffer was added on the cell. The cells were scraped from the plates and transferred to Eppendorf tubes, followed by centrifugation at 13,000 rpm for 2 min. Mixing100µl of the supernatant to 100µl of luciferase buffer (lysis buffer containing 1mM ATP, 1% (v/v) BSA and 0.2 mM luciferin substrate) in the cuvette. The relative light units were measured using a luminometer (F12 luminometer, Berthold detection system).

2.6 Immunofluorescent Microscopy

2.6.1 Transfection of yellow fluorescent protein (YFP)-tagged IKKα or YFP-tagged p65 DNA

HUVECs were seeded in 12 wells plates, and allowed to reach between 60-70% confluency. 1µg of DNA were then added into 1ml of media per well. FuGENE® HD Transfection Reagent was mixed with 2.5µl per 1µg of DNA. Cells were incubated with 1ml of media which contain transfection reagent/DNA overnight and were replaced with fresh media next day. Cells were left to recover for another 24h followed by stimulation with TNF-α (10ng/ml) for indicated time periods.

2.6.2 Immunofluorescent Microscopy

HUVECs were transfected with YFP-tagged IKKα or p65 in 12 well plates that contained glass coverslips and then stimulated as appropriate, coverslips were washed in ice cold PBS twice prior to fixation with ice cold methanol (500µl) at room temperature in dark for 10 min. After fixation, cells were washed twice with ice cold PBS and cell were incubated with 500µl of DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (1:2000) for 5 min. After incubation, cells were washed with PBS twice again. Mowiol was added on the slide and the coverslips were placed on top of the slide. Slides were kept away from light and left overnight before microscopy. Cells were visualized using a Nikon TE300-E upright epifluorescence microscope and imaged at x100 and x40 magnification using an oil-

immersion Plan Fluor objective lens. Images were collected using a digital Cool Snap-HQ CCD camera (Roper Scientific, Photometrics, Tucson, AZ). MetaMorph Imaging Series 7.0 (Molecular Devices Corp., Downington, PA, USA) was used for control of image acquisition, processing and modification of all image data. The background average statistical correction editing function in MetaMorph was used to produce background corrected images. This was achieved by determining the average background level of fluorescence from regions of interest drawn adjacent to cells expressing fluorescence.

2.7 Preparation of recombinant adenoviruses

2.7.1 Crude Adenoviral Lysates

Crude lysates of dominant negative inhibitory kappa B kinase alpha (DN-IKKα) and dominant negative inhibitory kappa B kinase beta (DN-IKKβ) virus were generated in HEK 293 cells, by infecting a 75cm^2 flask with 0.75µl of original stock virus. Flasks were incubated at 37°C, 5% CO_2, 95% air for 5-7 days until the cytopathic effect had been observed and the cells had started to detach from the surface of the flask. Cells were then removed from the flask and subjected to centrifugation (1500g for 5 min). The supernatant was removed and pellet was washed twice with PBS and centrifuged at 1500g for 5 min again. The collected pellet was then resuspended in 1ml of HE buffer (10mM HEPES pH 7.5, 1mM EDTA), frozen in liquid nitrogen and thawed in a 37°C water bath. This was repeated a further 2 times. After the third cycle, cells were centrifuged at 1500g for 5min to pellet the debris and the supernatant, which constitute the crude adenoviral lysates, was collected in sterile tube and stored at -80°C until required.

2.7.2 Generation and Purification of High-Titre Stocks of Adenovirus

The high-titre stocks of adenovirus were generated by large scale amplification of the crude adenoviral lysates described in section 2.8.1. 21 T175 flasks of HEK 293 cells were grown to approximately 70-80% confluency.1 flask was used as negative control, while the 20 flasks of medium were then changed to 2% FCS medium (24ml) before receiving 1ml of medium of diluted virus (100µl crude adenoviral lysates added to 25ml of HEK 293 medium). These flasks were incubated at 37°C, 5% CO2 until the cytopathic effect had occurred and cells had detached from the flask. The cells were then collected together with medium from the flasks and pelleted by centrifugation in a 50ml centrifuge tube at 1500g for 5 min. Majority of the

supernatant was removed in a sterile centrifuge tube and stored at 4°C until use. Cells were resuspended in 25 ml of supernatant and frozen in liquid nitrogen and thawed in at 37°C water bath, this was repeated a further 2 times. Subsequently, cells were mixed by vortexing after each thaw. Following the third cycle, cells were pelleted by centrifugation at 1500g for 5min. Supernatant was collected in a sterile tube while the pellet was discarded. A BD Adeno-X virus purification kit from Clontech Laboratories was used for the adenovirus purification. The supernatant was subject to filtering and then incubation with benzonase (25 units/µl) (50mM Tris-HCl pH8.0, 20mM NaCl, 2 mM $MgCl_2$, 50% glycerol) at 37°C for exact 30 min. Meanwhile, 1 x dilution buffer and 1 x washing buffer were prepared by diluting the provided 5 x buffers with sterile H_2O, according to the manufacturing instructions. The benzonase treated filtrate was mixed with an equal volume of 1 x dilution buffer and passed through BD Adeno-X syringe-Filter followed by washing with 1 x washing buffer. Adenovirus was later eluted from the mega filter using elution buffer and the elutate collected in sterile Eppendorf tubes and aliquots then stored at -80°C until titration.

2.7.3 Titration of Adenovirus by End-Point Dilution

Titration of the end point dilution method was used to quantify virus production (Nicklin and Baker, 1999). Serial dilutions of the adenovirus were applied to a 96 well plate of HEK 293 cells that reached approximately 70-80% confluency. The plate was incubated at 37°C, 5% CO_2 overnight after which the medium containing adenovirus was replaced with fresh medium. After that the plate was incubated for 10 days at 37°C, 5% CO_2 until the cytopathic effect of the virus had reached a steady state. Wells containing plaques were counted and the titre of adenovirus stock in terms of plaque-forming unit (pfu) was calculated as in the example shown below.

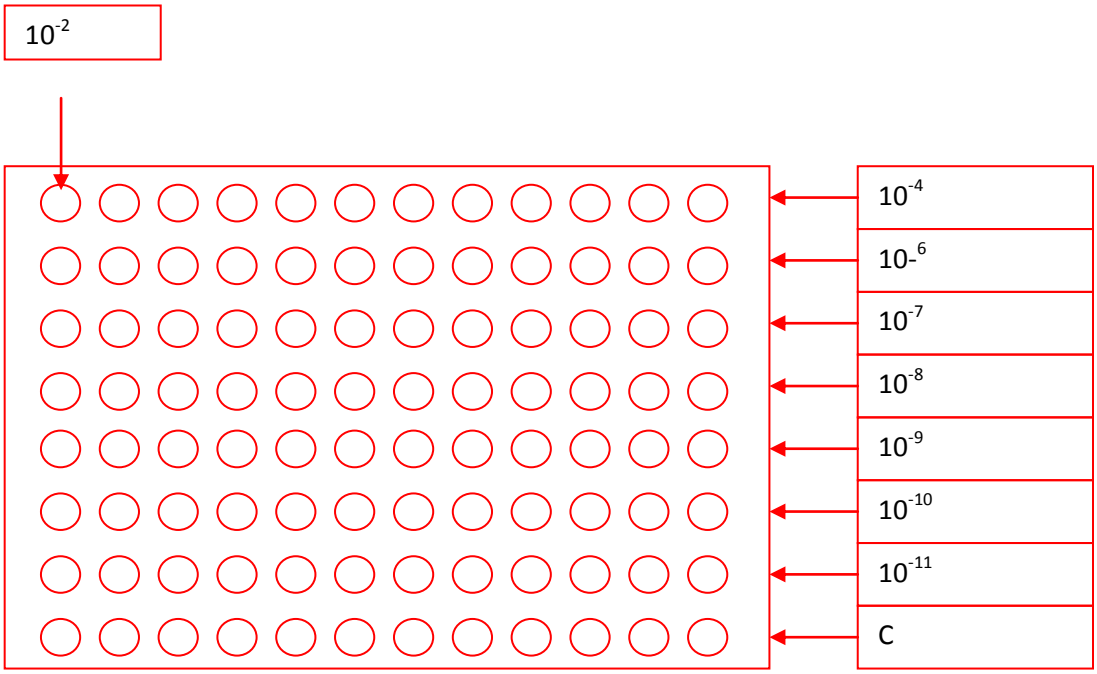


Figure 2.1 the End-Point Dilution for adenovirus titration. A series of concentrations of the adenovirus were applied on each of the row. The cells were observed in Day 10. The number of cells showed cytopathic effect would be counted in each of the concentration for further calculation.

Calculation of titre value

Using the two lowest concentrations for rest of calculation which present with plaques (although conc 1must be over 50).

Example.

	Concentration	Plaque present wells	Percentage
Concentration 1	10^{-9}	6/10	60
Concentration 2	10^{-10}	2/10	20

$$\frac{\text{Percentage of conc1} - 50}{\text{Percentage of conc1- percent of conc2}}$$

$$\frac{60 - 50}{60 - 20} = 0.25$$

Log ID_{50}

Concentration 1 + (0.25 x -1)

$-9 + (0.25 \times -1) = -9.25$

⇨ $ID_{50} = \log -9.25$

⇨ $10^{-9.25}$

TCID

$1/10^{-9.25} = 10^{9.25}$

$10^{9.25}$ X dilution factor (10) = $10^{10.25}$

Shift log $10^{10.25} = 1.778 \times 10^{10}$

One TD50 = 0.7 plaque forming units

=> $1.778 \times 10^{10} \times 0.7 = 1.245 \times 10^{10}$ pfu/ml

Or 1.245×10^{7} pfu/ul

Figure 2.2 The calculation of the titre of the adenovirus. The titre of adenovirus would be used to determine the amount of adenovirus for each well.

2.7.4 Infecting HUVECs with Adenovirus

To establish the appropriate volume of adenovirus to add to cells in order to give an appropriate multiplicity of infection (MOI), cells were grown to approximate 50-60% confluency in 12 well plates. The cell number (10µl) was assessed by using a haemocytometer and the cell number in 1ml was determined by the formula:

Total amount of cells (10µl) x 10^3 / 4

The amount of adenovirus applied to the cells was determined by another formula:

(Pfu) x total number of cell (1ml)/ titre value of the adenovirus

An appropriate MOI of adenovirus (50-300pfu/cell) was added to the cells and incubated for 40h in normal growth medium before stimulation. After this period, cells were exposed to agonist, TNF-α or IL-1β for the indicated time periods.

2.8 Scanning Densitometry

Western Blots were scanned on an Epson Perfection 1640SU Scanner using Adobe photoshop 5.0.2 software. The captured images were then normalized to a control and quantified using Scion Image (Scion Corp., Maryland, USA).

2.9 Data Analysis

All data shown were expressed as mean ± s.e.m and were representative of at least three separate experiments. The statistical significance of differences between mean values from control and treated groups were determined by either one-tailed Student's Unpaired t-test or a two tailed one-way analysis of variance (ANOVA) with Dunnett's post test ($p<0.05$ was considered significant).

Chapter 3

Characterisation the role of IKKα in the regulation of Canonical and Non canonical NFκB pathways

3.1 Introduction

Emerging evidence indicates that IKKα plays a critical role in the regulation of both canonical and non-canonical NFκB pathway. Studies using knockout models and molecular manipulation supports the hypothesis that IKKα regulates gene transcription by a phosphorylation of a number of nuclear targets including histone H3 (Yamamoto et al., 2003) and CBP (Huang et al., 2007). Furthermore IKKα has been demonstrated to play a role in the regulation of p100 processing (Liang et al., 2006; Luftig et al., 2004 Muller and Siebenlist, 2003) and p65 phosphorylation (Solt et al., 2009). TNF-α induced IKKα phosphorylation has also been demonstrated in many different cell types (Discussed in chapter 1).

Despite however the evidence presented above, there are a number of issues to be considered when examining the role of IKKα. First of all is the difficulty in measuring IKKα activation within the cell. Our previous studies in the laboratory found the specificity of commercial phospho-IKKα antibodies to be problematic, results could not be obtained using Western blotting. Furthermore *in vitro* kinase activity following immunoprecipitation using specific IKKα antibodies is difficult to interpret due to co-immunoprecipitation of both IKKβ and IKKγ as part of the complex. However a number of studies have shown TNFα induced nuclear translocation of IKKα in MEF and HeLa cells and this may also be a feature of endothelial cells. Therefore in the first part of the chapter, TNFα stimulated nuclear translocation of IKKα in HUVECs will be examined. Nuclear IKKα could possibly be a good marker for activation of both canonical and non-canonical NFκB pathways.

Another feature of IKKα which makes defining its role in endothelial cell function difficult is the potential for it to function not only as a kinase but also as a scaffolding protein. Thus techniques designed to inhibit both the kinase activity of IKK and also its expression need to be considered. Therefore in this chapter, we also tested an adenoviral version of dominant negative IKKα and both siRNA and shRNA constructs, the latter again as adenovirus.

3.2 Characterisation of nuclear IKKα in HUVECs

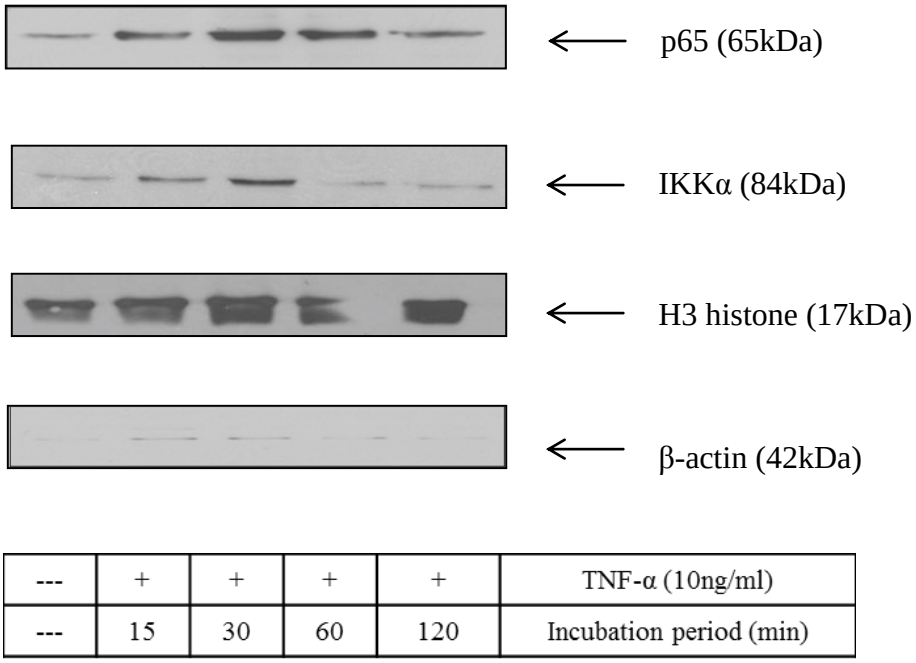
The kinetics of TNF-α induced nuclear translocation of IKKα was examined using two approaches. Firstly nuclear extracts were generated using sub-cellular fractionation (see chapter 2.4) and secondly, cells were transfected with epitope-tagged IKKα.

3.2.1 Subcellular fractionation to determine the nuclear translocation of IKKα and p65

HUVECs were incubated with TNF-α (10ng/ml) to induce nuclear translocation of IKKα. Two methods were employed to isolate nuclei, one a crude method used in routine electrophoretic mobility shift assay (EMSA) studies within the laboratory (Goh et al., 2009, MacKenzie et al., 2007) and the second a modified approach (Solt et al., 2009). Both gave similar results, however the standard method gave more protein to use in immunoblots.

The expression of IKKα in the nuclear fraction of HUVECs at various time points following TNF-α stimulation is shown in figure 3.1. IKKα levels in the nucleus increased by 15 min and reached a maximum at 30 min of approximately 4 folds of basal values. However, translocation was transient, levels returned to basal values by 60 min. TNF-α also stimulated an expected increase in nuclear expression of p65 suggesting that the cells had been effectively stimulated. The recovery of histone H3, a nuclear marker, indicated consistency of loading. The expression of the cytoplasmic protein, β-actin, was used as a negative control to confirm the relative purity of the nuclear samples.

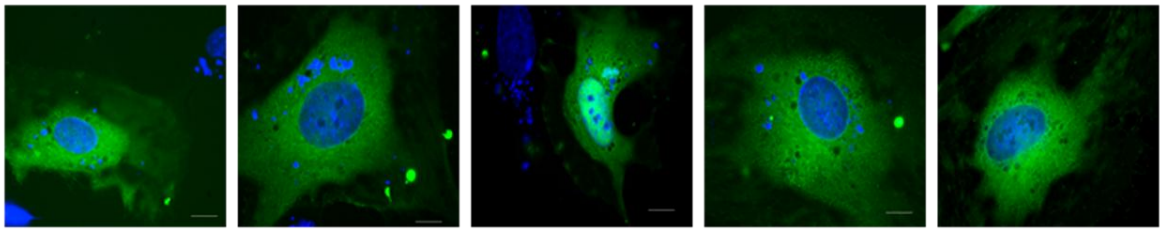
To confirm the results obtained with subcellular fractionation, immunofluorescence staining was performed. Preliminary studies demonstrated that staining of endogenous IKKα with commercially available antibodies gave a strong non-specific background and could not be used routinely. Thus, yellow fluorescence protein (YFP) tagged IKKα DNA was transfected into HUVECs, followed by direct microscopy. In figure 3.2, YFP-tagged IKKα was stained in green, while the nucleus was stained blue by Dapi. In the control, non-stimulated condition, YFP-IKKα was retained within the cytoplasm. In the majority of cells, YFP-IKKα transmigrated into the nucleus following 30 min of stimulation with TNF-α. Similar to results obtained by sub-cellular fractionation, nuclear IKKα gradually returned to a cytosolic location within 2 hours. YFP-tagged p65 was used as positive control, TNF-α induced nuclear translocation of p65 demonstrated that the cells had been effectively stimulated.



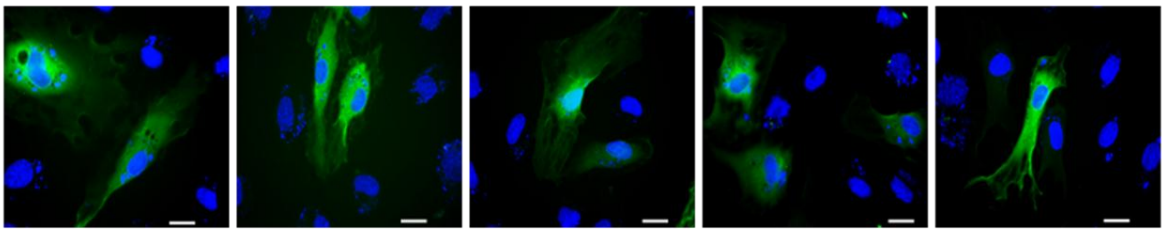
---	+	+	+	+	TNF-α (10ng/ml)
---	15	30	60	120	Incubation period (min)

Figure 3.1: TNF-α induced nuclear translocation of IKKα in HUVECs using sub cellular fractionation. Cells were stimulated with 10ng/ml of TNF-α for various time periods. Nuclear lysates were prepared, separated by SDS-PAGE, and then assessed for IKKα (84kDa), p65 (65kDa), H3 histone (17kDa) and β-actin (42kDa) expression as outlined in section 2.4. The result is representative of 1 experiment.

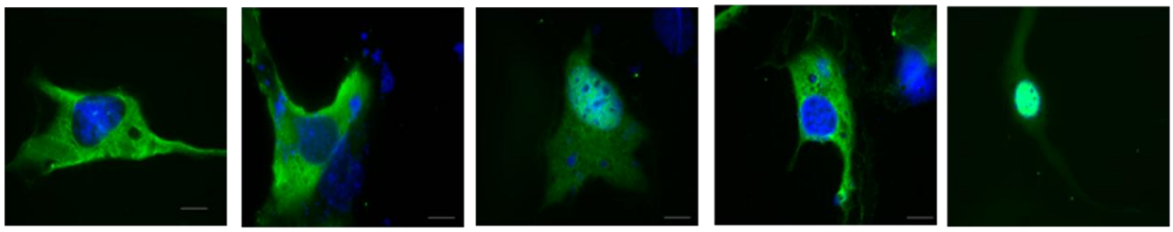
A



B



C



---	+	+	+	+	TNF-α (10ng/ml)
---	15	30	60	120	Incubation period (min)

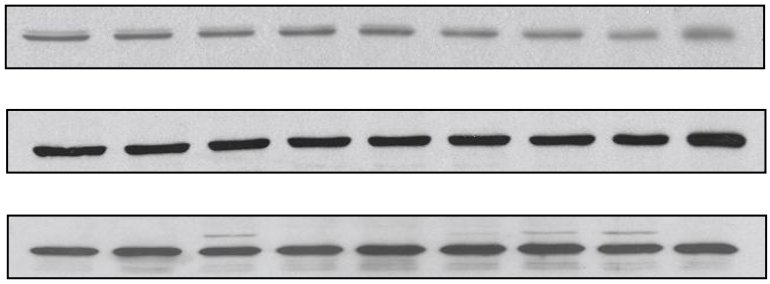
Figure 3.2: TNF-α induced nuclear translocation of IKKα in HUVECs assessed by immunofluorescence. Cells were transiently transfected with 1μg/ml of YFP-IKKα or YFP-p65 for 24hr prior to stimulation with TNF-α (10ng/ml) as outlined in section 2.6.1, then followed by direct immunofluorescence as described previously (section 2.6.2). The nuclei were stained in blue colour and the YFP-tagged IKKα was in green colour. In Panels A and C, cells were viewed at x100 magnification, while cells in panel B were viewed at x40 magnification, using a Nikon EP-1 fluorescent microscope. The results are representative of 3 independent experiments.

3.3 Comparison of knockdown efficiency for Adv. shRNA IKKα and siRNA IKKα

Previously in the laboratory, we have used adenoviral versions of both IKKα and β (Goh et al., 2009; MacKenzie et al., 2007) to assess their roles in NFκB signalling and studies using these constructs are shown later in this chapter. However, because of limitations in their usefulness, we also sought to develop Adv. shRNA approaches to ablate endogenous IKKα as HUVECs are sensitive to the traditional transfection agents and show substantial toxicity. Figure 3.3 shows the efficiency of knockdown of endogenous IKKα using Adv. shRNA IKKα and as an alternative using standard siRNA IKKα. Panel A is a concentration curve for both shRNA IKKα and as a comparator, IKKβ. HUVECs were infected with shRNA IKKα or β for 96h, although similar results were obtained following shorter periods of infection. Increasing the concentration of shRNA IKKα up to a maximal concentration (1000pfu) showed no significant difference upon endogenous IKKα expression in comparison to control. Similarly, a maximum concentration (1000pfu) of shRNA IKKβ also failed to knockdown endogenous IKKβ expression in HUVECs. The presence of LacZ did not affect the expression of either IKKα or IKKβ.

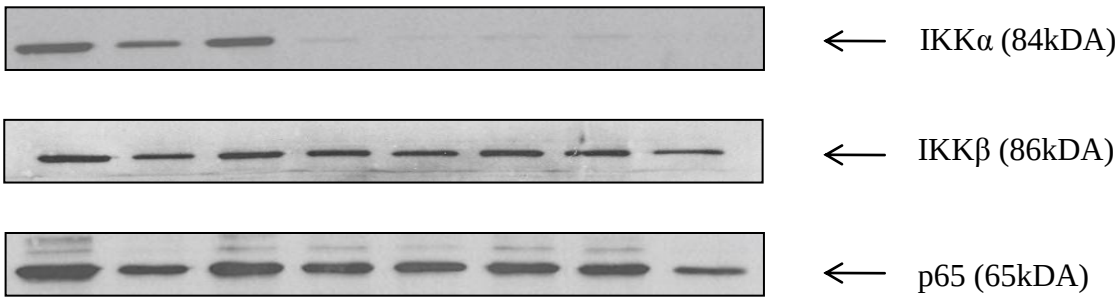
In panel B, HUVECs were transfected with siRNA IKKα at increasing concentrations up to a maximum of 200nM. Negative control non-targeting siRNA acted as a scrambled sequence for IKKα and showed a minimal effect on endogenous IKKα expression. Surprisingly, the lowest concentration (100nM) of siRNA IKKα effectively knocked down endogenous IKKα expression, reducing levels by approximately 90%. Furthermore siRNA IKKα even at maximum concentration was without effect on IKKβ levels suggesting a selective effect. Total p65 was used as a loading control and demonstrated that either non-targeting or IKKα do not cause a toxic effect in HUVECs. Overall this result confirmed that siRNA IKKα was effective and selective and also induced a minimal cytotoxic effect to the cell. Therefore, siRNA IKKα was used for further studies.

A



---	---	500	700	1000	---	---	---	---	shRNA IKKα (pfu)
---	---	---	---	---	300	500	700	1000	shRNA IKKβ (pfu)
---	+	---	---	---	---	---	---	---	LacZ (1000pfu)

B



---	---	---	100	125	150	175	200	siRNA IKKα (nM)
---	+	---	---	---	---	---	---	NT (200nM)
---	+	+	+	+	+	+	+	Fugene (3.7µL)

Figure 3.3: The effect of Adv. shRNA IKKα and siRNA IKKα on IKKα expression in HUVECs. A) Cells were infected with increasing concentration of Adv. shRNA IKKα or shRNA IKKβ for 96h and B) Cells were transfected with non-targeting scramble sequence or siRNA IKKα up to a concentration of 200nM. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for IKKα (84kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3. The results are representative of 3 independent experiments.

3.3.1 TNF-α induced IκB-α loss in HUVECs in a time dependent manner

It has been shown previously in the laboratory that TNF-α induced a loss in cellular IκB-α in HUVECs leading to the activation of the NFκB pathway (discussed in Section 1.3.3). Therefore the aim of this initial experiment was to confirm the optimal time period for TNF-α induced IκB-α loss. In figure 3.4, IκB-α degradation was observed 5min after TNF-α (10ng/ml) stimulation and levels reached a minimum at 15min (% basal expression = 1.37%) and was sustained over 30min (% basal expression = 1.10%). Subsequently, the expression of IκB-α gradually recovered, returning towards basal values by 90min (% basal expression = 21.5%).

3.3.2 The effect of Adv. DN-IKKβ in the regulation of TNF-α induced IκB-α degradation in HUVECs

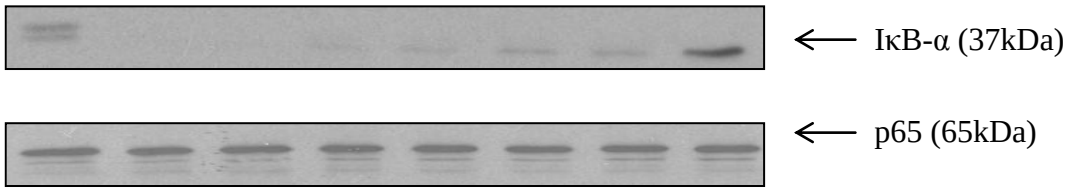
A number of studies have shown that IKKβ plays an intimal role in the regulation of the canonical NFκB pathway (Discussed in Section 1.3.3.8.1). Figure 3.5 shows a concentration curve for the effect of Adv. DN-IKKβ on TNF-α induced IκB-α degradation in HUVECs. HUVECs were infected with increasing concentrations of Adv. DN-IKKβ 40h prior to stimulation with TNF-α (10ng/ml). As expected, TNF-α induced IκB-α degradation after 15 min (% basal IκB-α expression = 6.21%, n = 1). Whilst this response was not altered in the presence of β-galactosidase (LacZ), the extent of IκB-α degradation was greatly reduced (% basal IκB-α expression = 73.7%, n = 1) following overexpression of DN-IKKβ. Both DN-IKKβ and the LacZ did not affect the expression of p65, this also indicated loading of each sample was equal.

3.3.3 Characterisation of the role of IKKα in the regulation of TNF-α induced IκB-α degradation in HUVECs

The role of IKKα in the regulation of the canonical NFκB pathway can be cell type dependent. Evidence has shown IKKα plays no role in the IκB-α loss in HeLa cells (Hu et al., 1999), vascular smooth muscle cells (MacKenzie et al., 2007) and MEF (Solt et al., 2009), whereas IKKα does play a role in the regulation of IκB-α loss in HT-29 cells (DiDonato et al.,1997) The following experiment was performed to determine whether IKKα shares similar characteristics with IKKβ in the regulation of the canonical NFκB pathway. Figure 3.6 shows a concentration curve for the effect of Adv. DN-IKKα on TNF-α induced IκB-α loss in

HUVECs. HUVECs were infected with Adv. DN-IKKα 40h prior to stimulation with TNF-α (10ng/ml). As expected, TNF-α significantly induced IκB-α degradation after 15min (% basal IκB-α expression = 5.43%). In contrast, and unlike IKKβ, increasing the concentration of Adv DN-IKKα did not reverse IκB-α loss. Similar to previous experiments, the agonist induced response was not altered in the presence of LacZ. The expression of p65 was used as a loading control and indicated that the concentration of protein in each sample was equal.

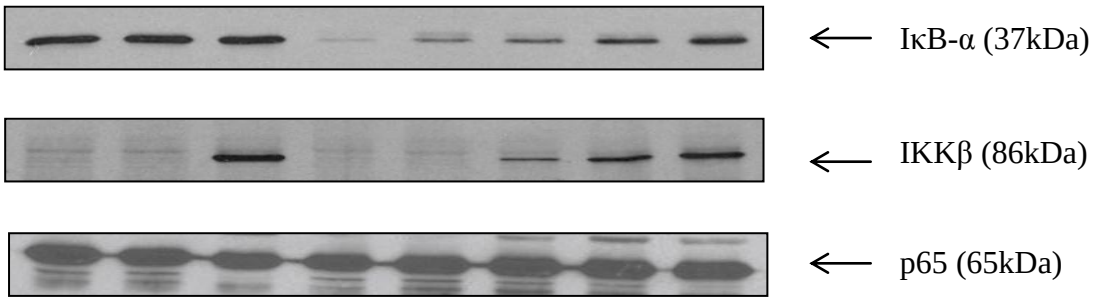
In order to confirm the results obtained with individual treatments, HUVECs were simultaneously pre-treated with a maximum concentration of either Adv. DN-IKKα or β for 40h prior to stimulation with TNF-α (10ng/ml). Figure 3.7 demonstrated that TNF-α significantly induced IκB-α degradation after 15min (% basal IκB-α expression = 12.26 ± 4.57, n = 3, $P<0.01$). Whilst this response was not altered in the presence of DN-IKKα, the extent of IκB-α degradation was significantly reduced (% basal IκB-α expression = 88.9 ± 5.38, n = 3, $P<0.01$) following overexpression of Adv. DN-IKKβ. This confirmed the selective effect of Adv. DN-IKKβ within the canonical NFκB pathway.



+	+	+	+	+	+	+	---	TNF-α (10ng/ml)
5	15	30	45	60	75	90	---	Incubation period (min)

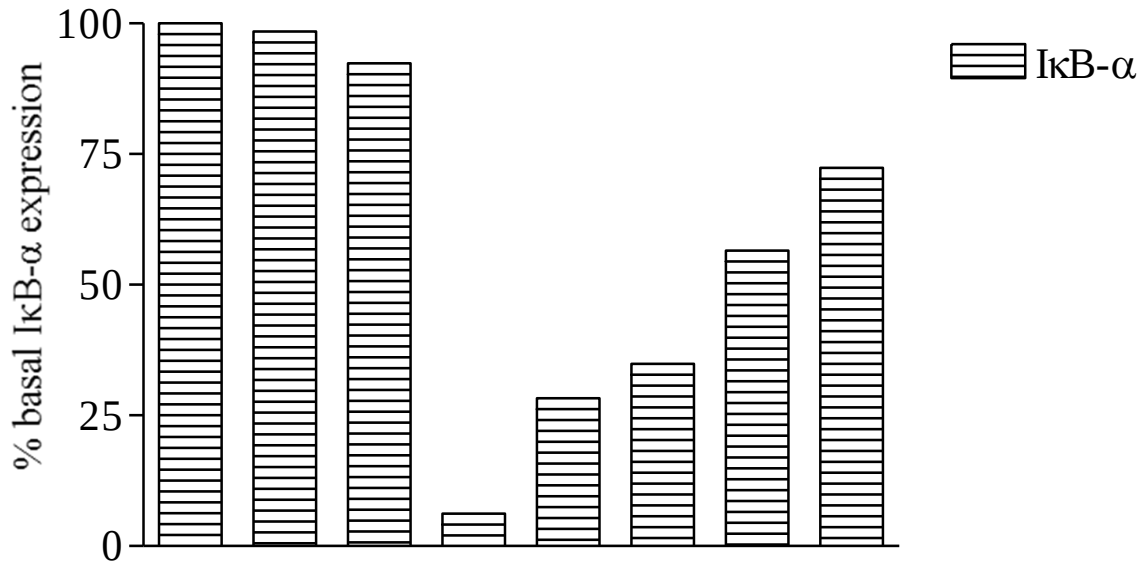
Figure 3.4: TNF-α-mediated IκB-α loss in HUVECs. Cells were stimulated with TNF-α (10ng/ml) as indicated. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for IκB-α (37kDa) and p65 (65kDa) as outlined in Section 2.3. The result is representative of 1 experiment.

A



---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	300	---	---	100	200	300	DN-IKKβ (pfu)
---	+	---	---	+	---	---	---	LacZ (300pfu)

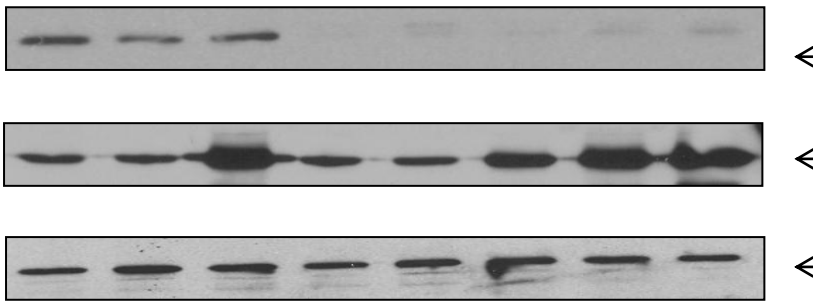
B



---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	300	---	---	100	200	300	DN-IKKβ (pfu)
---	+	---	---	+	---	---	---	LacZ (300pfu)

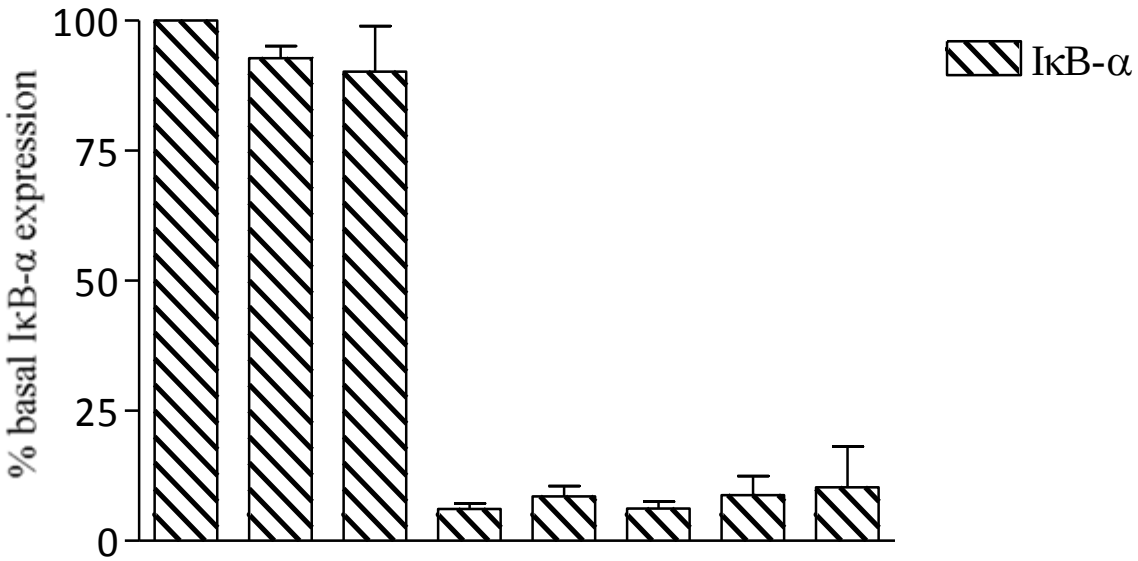
Figure 3.5: The effect of Adv DN-IKKβ upon TNF-α-mediated IκB-α loss in HUVECs. Cells were infected with either 300 pfu of Adv. LacZ or increasing pfu of Adv. DN-IKKβ for 40h prior to stimulation with TNF-α (10ng/ml) for a further 15min. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) IκB-α (37kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3. B) Blots were quantified for % basal IκB-α expression by scanning densitometry. The result is representative of 1 experiment.

A



---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	300	---	---	100	200	300	DN-IKKα (pfu)
---	+	---	---	+	---	---	---	LacZ (300pfu)

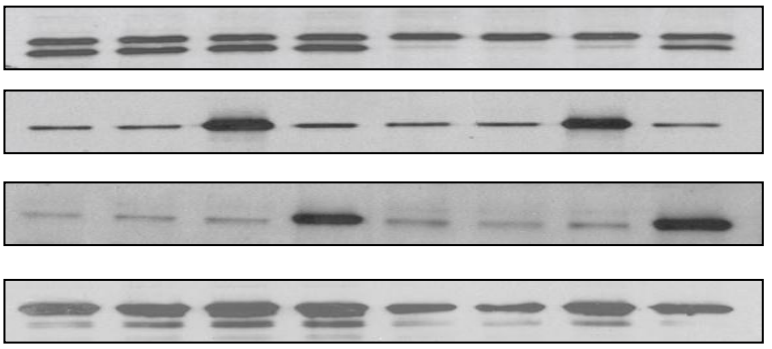
B



---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	300	---	---	100	200	300	DN-IKKα (pfu)
---	+	---	---	+	---	---	---	LacZ (300pfu)

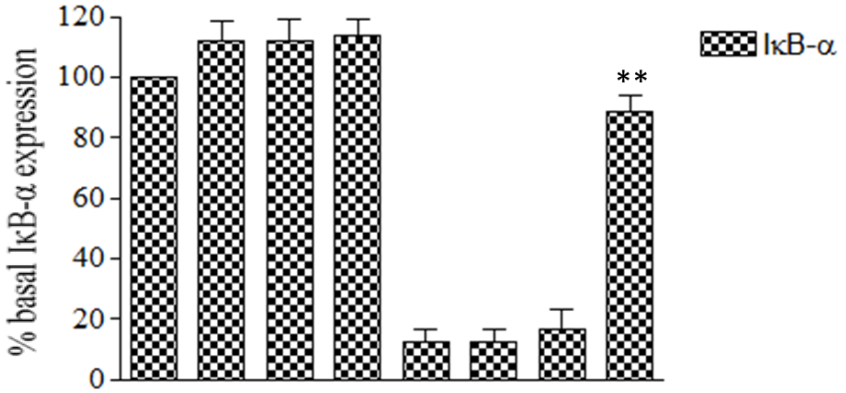
Figure 3.6: The effect of Adv. DN-IKKα upon TNF-α-mediated IκB-α loss in HUVECs. Cell were infected with Adv DN-IKKα or LacZ for 40h prior to stimulation with TNF-α (10ng/ml) for a further 15min. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) IκB-α (37kDa), IKKα (84kDa) and p65 (65kDa) as outlined in section 2.3. B) Blots were quantified for % basal IκB-α expression by scanning densitometry, each value represents the mean ± S.E.M from 3 independent experiments.

A



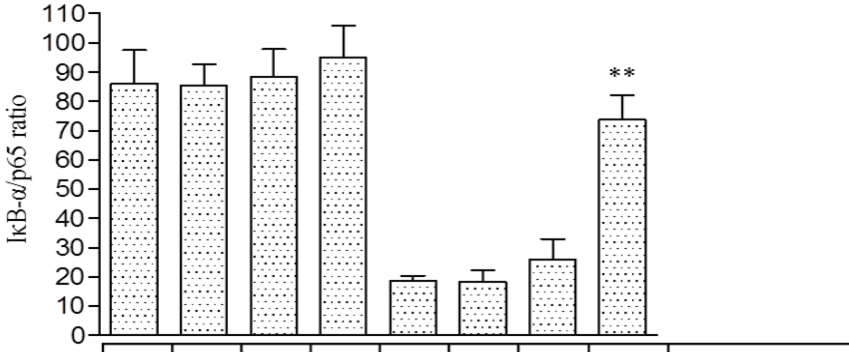
---	---	---	---	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	+	---	DN-IKKα (300pfu)
---	---	---	+	---	---	--	+	DN-IKKβ (300pfu)
---	+	---	---	---	+	---	---	LacZ (300pfu)

B



---	---	---	---	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	+	---	DN-IKKα (300pfu)
---	---	---	+	---	---	--	+	DN-IKKβ (300pfu)
---	+	---	---	---	+	---	---	LacZ (300pfu)

C



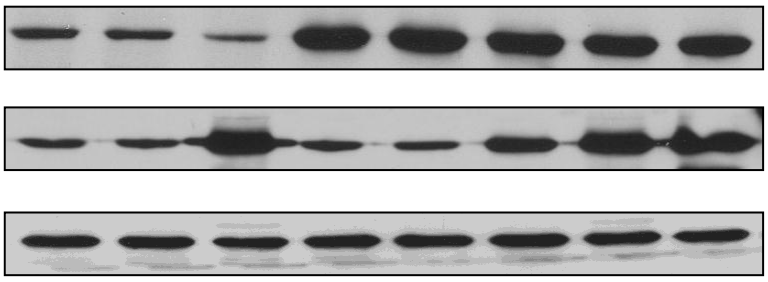
---	---	---	---	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	+	---	DN-IKKα (300pfu)
---	---	---	+	---	---	--	+	DN-IKKβ (300pfu)
---	+	---	---	---	+	---	---	LacZ (300pfu)

Figure 3.7: The effect of Adv. DN-IKKα upon TNF-α-mediated IκB-α degradation in HUVECs. Cells were infected with Adv. DN-IKKα, β or LacZ for 40h prior to stimulation with TNF-α (10ng/ml) for 15min. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) IκB-α (37kDa), IKKα (84kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) % basal IκB-α expression and c) IκB-α/p65 ratio by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments. *P<0.05, **P<0.01 compared with agonist stimulated control.

3.3.5 The effect of Adv. DN-IKKα in the regulation of TNF-α induced p65 phosphorylation in HUVECs

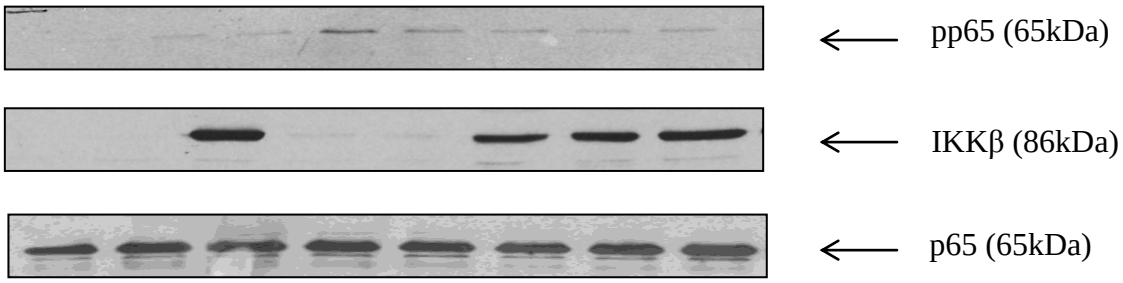
Similar to the previous experiment, HUVECs were infected with either DN-IKKα or β for 40h prior to stimulation with TNF-α (10ng/ml). In figure 3.8, panel A shows that TNF-α induced a 3 to 5 fold increase in the phosphorylation of p65 after 15min of stimulation. This response was not altered in the presence of 300 pfu of Adv. DN-IKKα, in contrast, increasing concentrations of Adv. DN-IKKβ (see panel B) reduced phosphorylation of p65. Maximum concentrations of LacZ did not affect p65 phosphorylation and total p65 was used as a loading control.

A



---	---	300	---	---	100	200	300	DN-IKKα (pfu)
---	---	---	---	+	---	---	---	LacZ (300pfu)
---	---	---	+	+	+	+	+	TNF-α (10ng/ml)

B



---	---	300	---	---	100	200	300	DN-IKKβ (pfu)
---	---	---	---	+	---	---	---	LacZ (300pfu)
---	---	---	+	+	+	+	+	TNF-α (10ng/ml)

Figure 3.8: The effect of Adv. DN-IKKα or β upon TNF-α-mediated p65 phosphorylation in HUVECs. Cells were infected with A) Adv. DN-IKKα, B) Adv. DN-IKKβ or LacZ for 40 hours prior stimulation with TNF-α (10ng/ml) for 15min. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for p-p65 (65kDa), IKKα (84kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3

3.3.6 Lack of effect of Adv. DN-IKKα in IL-1β activated canonical NFκB pathway in HUVECs

Similar to TNF-α, IL-1β can also induce activation of the canonical NFκB pathway. However the effect of IL-1β is thought to be mediated by a different upstream mechanism, this would still lead to activation of the IKK complex, subsequent IκB-α degradation and p65 nuclear translocation (discussed in Section 1.3.3.8). In this section, the role of IKKα in IL-1β induced NFκB activation was assessed and the result compared to TNF-α induced responses.

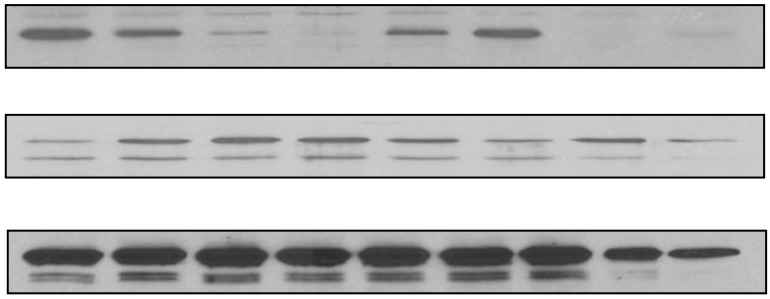
3.3.7 Characterisation of the effect of IL-1 β on the canonical NFκB pathway in HUVECs

Figure 3.9 shows a time course of IκB-α degradation (panel A) and phosphorylation of p65 (panel B) induced by of IL-1β. Following stimulation of HUVECs with 10ng/ml of IL-1β, IκB-α levels decrease rapidly reaching a minimum value between 10 and 30 min (% basal IκB-α expression = 17.3% and 10.6% respectively). As with TNF-α, degradation was transient with IκB-α returning to basal values by 120 min. This response compared well with the positive control, TNF-α, which gave maximal degradation between 15 and 30min. In addition, IL-1β induced p65 phosphorylation. This response started as early as 5min and reached maximum at 30min of stimulation. Figure 3.9 shows a concentration response curve for IL-1β induced IκB-α degradation and p65 phosphorylation. Both parameters increased in a concentration dependent manner between 1 and 10ng/ml at which the maximal response was obtained. However, the curve was very steep and concentration curves could not be easily quantified. Nevertheless, a concentration of 10ng/ml of IL-1β was used for subsequent experiments.

3.3.8 The effect of Adv. DN-IKKα and β on IL-1β induced NFκB activation in HUVECs

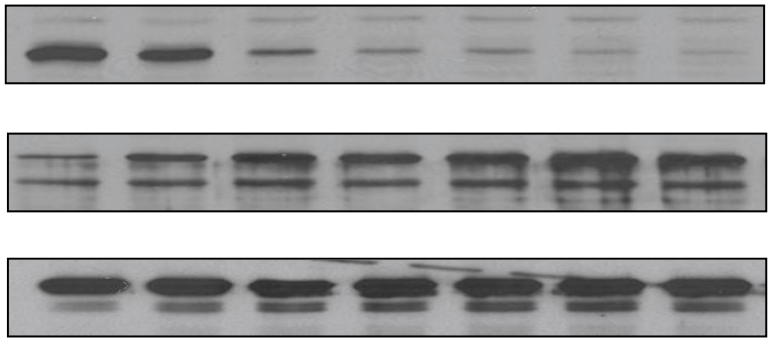
In figure 3.10, HUVECs were infected with Adv. DN-IKKα or β for 40h prior to stimulation with IL-1β. Using 10ng/ml of IL-1β resulted in a marked stimulation of IκB-α degradation after 30min (% basal IκB-α expression = 23.7%). Overexpression of Adv. DN-IKKα up to 300pfu or the lacZ control at the same concentration failed to affect the IL-1β induced loss in IκB-α. Similar results were obtained for IL-1β induced p65 phosphorylation, Adv. DN-IKKα was without effect.

A



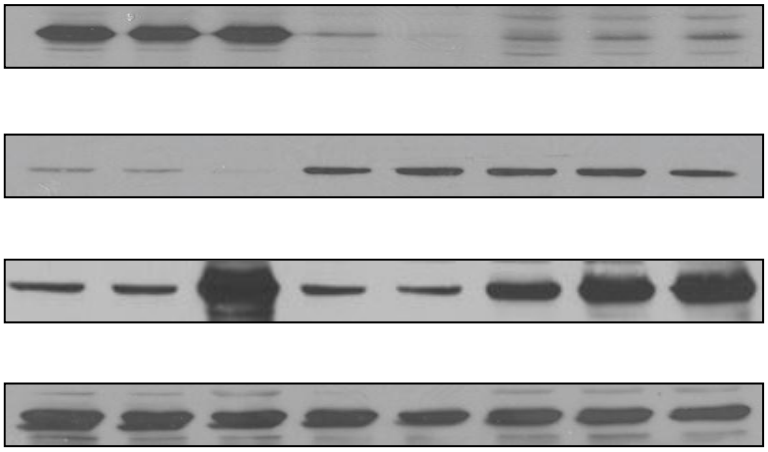
---	+	+	+	+	+	---	---	IL-1β (10ng/ml)
---	---	---	---	---	---	+	+	TNF-α (10ng/ml)
---	5	10	30	60	120	15	30	Incubation period (min)

B



---	1	5	10	20	30	---	IL-1 β (ng/ml)
---	---	---	---	---	---	10	TNF-α (ng/ml)

Figure 3.9: Time course and concentration dependency for IL-1β mediated IκB-α degradation and p65 phosphorylation. A) Cells were stimulated with IL-1β (10ng/ml) or TNF-α (10ng/ml) for the indicated time points. B) Cells were stimulated with increasing concentrations of IL-1β or 10ng/ml of TNF-α for 15 and 30min respectively. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for IκB-α (37kDa) degradation, phosphorylation of p65 (65kDa) and total p65 (65kDa) as outlined in section 2.3. The results are representative of 3 independent experiments



---	---	---	+	+	+	+	+	IL-1 β (10ng/ml)
---	---	+	---	---	100	200	300	DN-IKKα (300pfu)
---	+	---	---	+	---	---	---	LacZ (pfu)

Figure 3.10: The effect of Adv. DN-IKKα upon IL-1β mediated IκB-α degradation and p65 phosphorylation in HUVECs. Cells were infected with Adv. DN-IKKα or LacZ for 40h prior to stimulation with IL-1β (10ng/ml) for 30 min. Whole cell lysates were prepared, separated by SDS PAGE and assessed for a) IκB-α (37kDa), p-p65 (65kDa), IKKα (84kDa) and p65 (65kDa) as outlined in section 2.3.

3.4 The role of IKKα in the regulation of the non-canonical NFκB pathway

The processing of p100 is believed to be a critical up-stream activation step in the non-canonical NFκB pathway in which the formation of p52 allows NFκB (RelB/p50) to translocate into the nucleus. The literature reviewed in chapter 1 suggests that IKKα is the only IKK member to regulate p100 processing. Therefore the aims of the subsequent experiments are to investigate the effect of TNF-α on p100 processing in HUVECs and determine the role of IKKα in the regulation of this pathway.

3.4.1 Characterisation of the effect of TNF-α on the non-canonical NFκB pathway in HUVECs

Figure 3.11 panels A and B show an extended time course over 24h, using 2 blots, for TNF-α induced p100 processing. The response was found to be very slow with a lag period of 4 to 6h before an increase in p52 formation was observed. A maximum response was obtained by 8h and remained at these levels for the remainder of the time course. Interestingly, p100 expression also increased with similar kinetics to that observed for p52 formation. Again maximal expression was achieved at 24h of TNF-α stimulation (Fold stim = 3.69)

Figure 3.12 shows a concentration curve for TNF-α induced p100 processing. TNF-α stimulated the formation of p52 over the low ng/ml concentration range with a maximal response of 3.84 fold obtained at 10ng/ml. In contrast, p100 induction seemed to be maximal by 3ng/ml of TNF-α, however this difference was not investigated further. The experiment in figure 3.13 is similar to figure 3.12 panel B, 20ng/ml of IL-1β was used instead of TNF-α as a stimulant. A very weak response was observed after 8h stimulation with IL-1β which was sustained for the remainder of the time course.

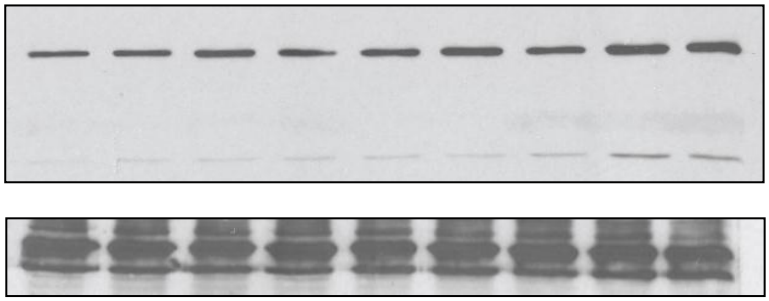
3.4.2 Characterisation of the effect of Adv. DN-IKKα in the non-canonical NFκB pathway in HUVECs

Initially, HUVECs were pre-treated with Adv. DN-IKKα or β for 40h prior to stimulation with 10ng/ml of TNF-α for a further 14h (Figure 3.14). As expected, TNF-α significantly increased the formation of p52 (Fold stim = 4.26 ± 0.44, n=3). However, whilst the response was not affected by overexpression of Adv. LacZ control, Adv. DN-IKKα significantly reduced p52 formation (Fold stim = 2.02 ± 0.50, n = 3, $p<0.01$). Furthermore, whilst p52 formation was significantly enhanced in the presence of Adv. DN-IKKβ (Fold stim = 5.91 ± 0.89, n = 3 $p<0.05$), incubation with the non-selective IKK inhibitor, BMS (10μM), significantly reduced the TNF-α response (Fold stim = 0.65 ± 0.074, n = 3, $p<0.01$). This inhibitory effect corresponded with a significant reduction in the expression of p100. Indeed, when the ratio of inhibition of p100 induction and p52 formation were compared (panel C), it was found that TNF-α stimulation and both Adv. DN-IKKα or β did not affect the p100:p52 ratio.

3.4.3 Characterisation of the effect of siRNA IKKα on the non-canonical NFκB pathway in HUVECs

In order to confirm the results obtained with the Adv. DN-IKKα, the effect of siRNA IKKα was examined in figure 3.15, HUVECs were pre-treated with siRNA IKKα or as a comparator infected with Adv. DN-IKKβ prior to stimulation with 10ng/ml of TNF-α for 14h. TNF-α significantly increased the formation of p52 (Fold stim = 4.26 ± 0.44, n=3, $p<0.01$). Although, the response was not affected by the presence of Adv. LacZ, siRNA IKKα significantly reduced TNF-α induced p100 processing (Fold = 1.62 ± 0.42, n = 3, $p<0.01$). In contrast, Adv. DN-IKKβ enhanced the response of TNF-α induced p100 processing (Fold= 6.38 ± 0.41, n = 3, $p<0.05$). Again, when the ratios of p52 formation were compared to the increase in p100 expression, it was found that TNF-α stimulation and both Adv. DN-IKKα or β did not affect the p100:p52 ratio.

A



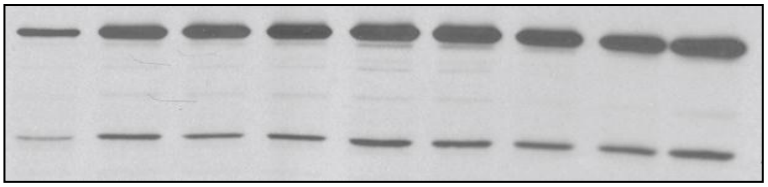
p100 (100kDa)

p52 (52kDa)

p65 (65kDa)

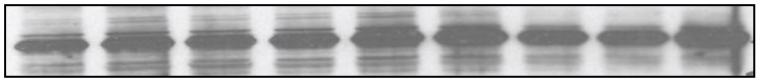
---	+	+	+	+	+	+	+	+	TNF-α (10ng/ml)
---	0.25	0.5	1	2	4	6	8	24	Incubation period (hours)

B



p100 (100kDa)

p52 (52kDa)



p65 (65kDa)

---	+	+	+	+	+	+	+	+	TNF-α (10ng/ml)
---	8	10	12	14	16	18	20	24	Incubation period (hours)

Figure 3.11: Time Course of TNF-α-mediated p100 processing in HUVECs. Cells were stimulated with TNF-α (10ng/ml) for A) a maximum 8h time period and B) an extended time period. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for p100 (100kDa) and p52 (52kDa) formation and total p65 (65kDa) as outlined in section 2.3. The results are representative of 1 independent experiment.

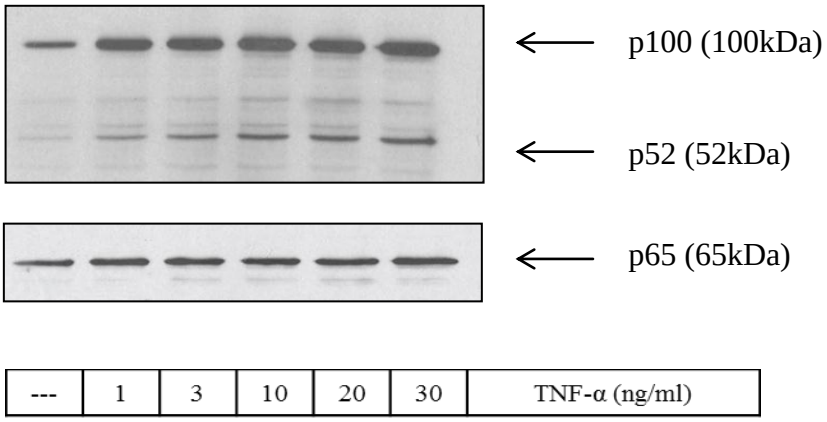
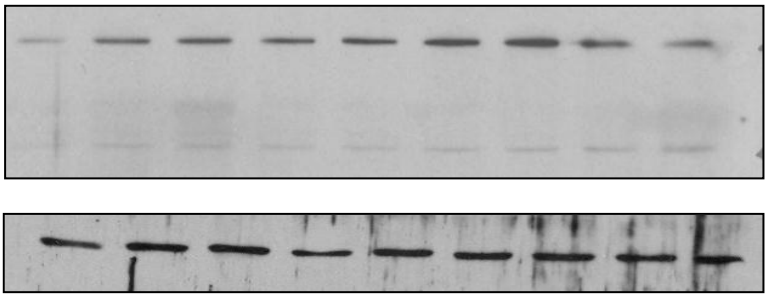


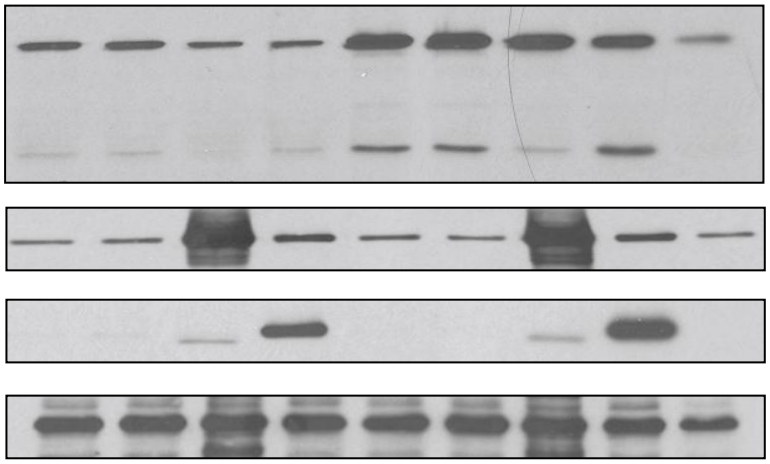
Figure 3.12: Concentration dependency of TNF-α-mediated p100 processing in HUVECs. Cells were stimulated with increasing concentrations of TNF-α for 14h. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for p100 (100kDa) and p52 (52kDa) formation and total p65 (65kDa) as outlined in section 2.3.



---	+	+	+	+	+	+	+	+	IL-1β (20ng/ml)
---	8	10	12	14	16	18	20	24	Incubation period (hours)

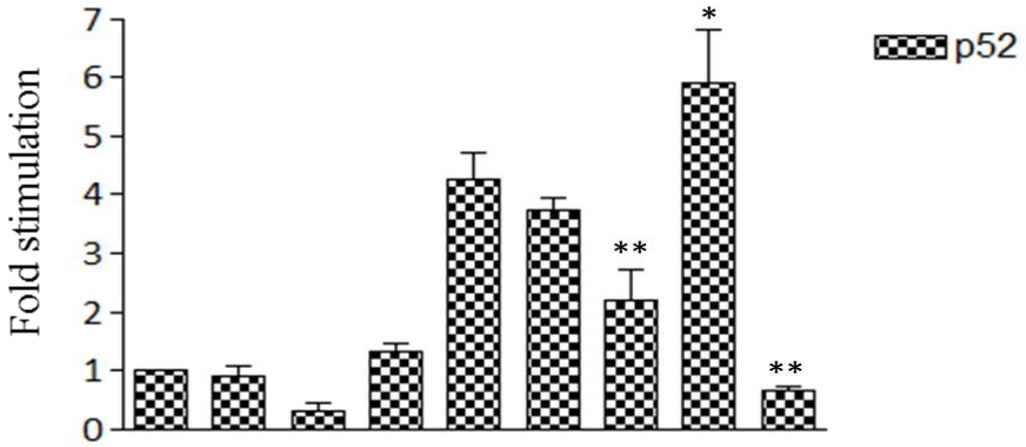
Figure 3.13: Time Course of IL-1β-mediated p100 processing in HUVECs. Cells were stimulated with IL-1β (20ng/ml) for the indicated time points. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for p100 (100kDa), p52 (52kDa) formation and total p65 (65kDa) as outlined in section 2.3.

A



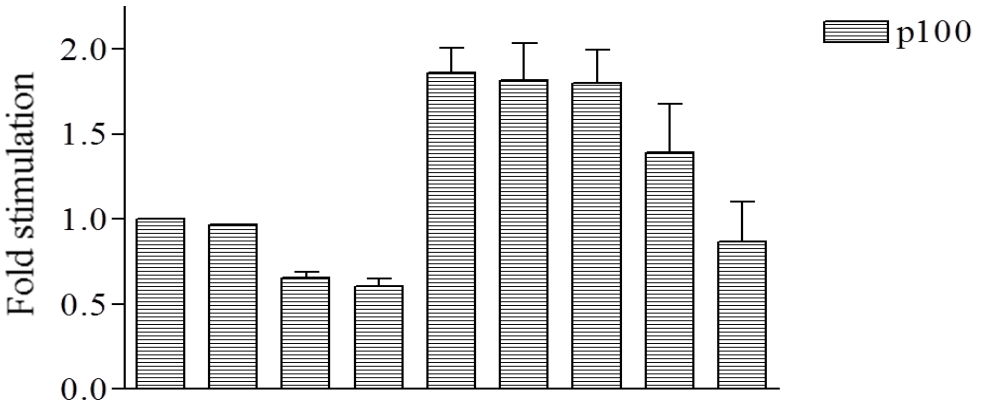
---	---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	+	---	---	DN-IKKα (300pfu)
---	---	---	+	---	---	---	+	---	DN-IKKβ (300pfu)
---	---	---	---	---	---	---	---	+	BMS (10μM)
---	+	---	---	---	+	---	---	---	LacZ (300pfu)

B



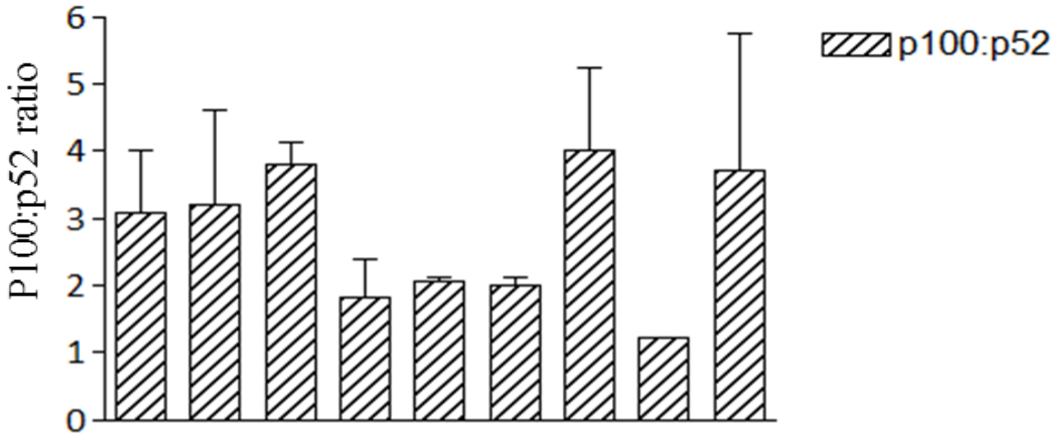
---	---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	+	---	---	DN-IKKα (300pfu)
---	---	---	+	---	---	---	+	---	DN-IKKβ (300pfu)
---	---	---	---	---	---	---	---	+	BMS (10μM)
---	+	---	---	---	+	---	---	---	LacZ (300pfu)

C



---	---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	+	---	---	DN-IKKα (300pfu)
---	---	---	+	---	---	---	+	---	DN-IKKβ (300pfu)
---	---	---	---	---	---	---	---	+	BMS (10μM)
---	+	---	---	---	+	---	---	---	LacZ (300pfu)

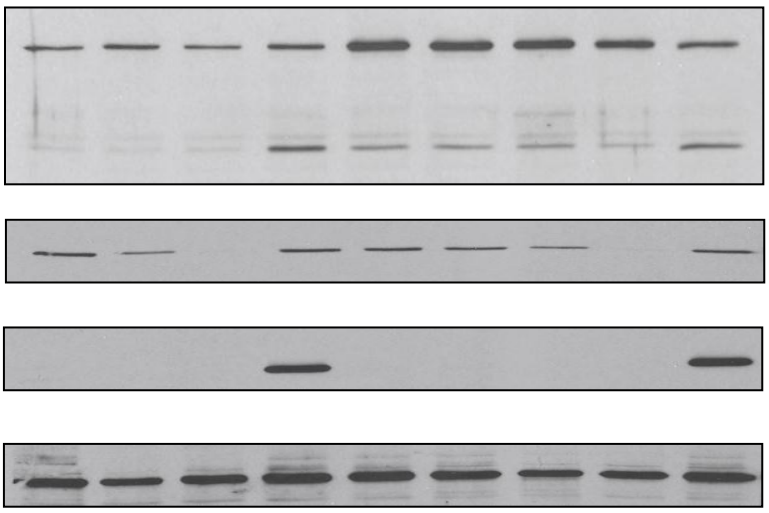
D



---	---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	+	---	---	DN-IKKα (300pfu)
---	---	---	+	---	---	---	+	---	DN-IKKβ (300pfu)
---	---	---	---	---	---	---	---	+	BMS (10μM)
---	+	---	---	---	+	---	---	---	LacZ (300pfu)

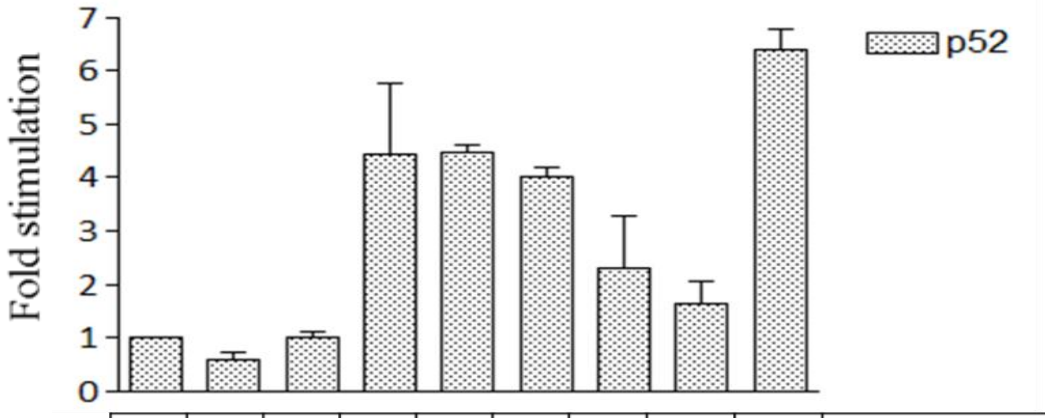
Figure 3.14: The effect of Adv. DN-IKKα or β upon TNF-α-mediated p100 processing in HUVECs. Cells were infected with Adv. DN-IKKα or β for 40h prior to stimulation with TNF-α (10ng/ml) for 14h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) p100 (100kDa), p52 (52kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) Fold stimulation of p52 and C) p100 are measured by scanning densitometry and D) The ratio of p100:p52, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments. *P<0.05, **P<0.01 compared with agonist stimulated control.

A



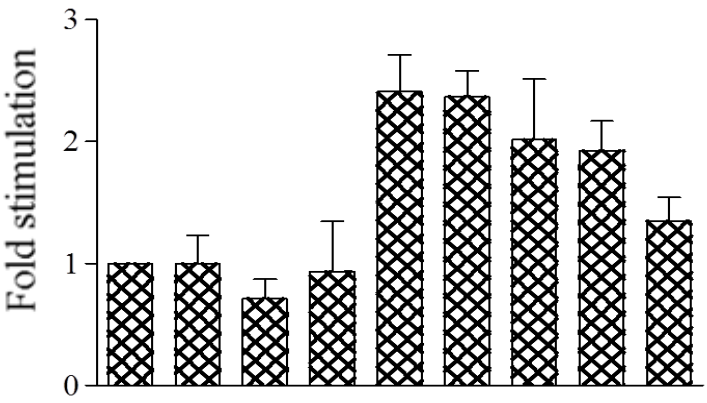
---	---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	---	+	---	siRNA IKKα (100nM)
---	---	---	+	---	---	---	---	+	DN-IKKβ (300pfu)
---	+	---	---	---	---	+	---	---	NT (100nM)
---	---	---	---	---	+	---	---	---	Laz (300pfu)

B



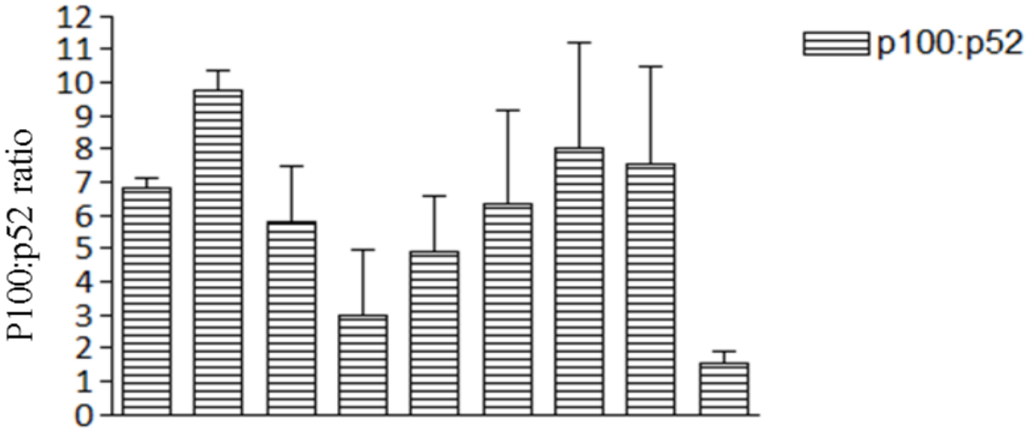
---	---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	---	+	---	siRNA IKKα (100nM)
---	---	---	+	---	---	---	---	+	DN-IKKβ (300pfu)
---	+	---	---	---	---	+	---	---	NT (100nM)
---	---	---	---	---	+	---	---	---	Laz (300pfu)

C



---	---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	---	+	---	siRNA IKKα (100nM)
---	---	---	+	---	---	---	---	+	DN-IKKβ (300pfu)
---	+	---	---	---	---	+	---	---	NT (100nM)
---	---	---	---	---	+	---	---	---	LacZ (300pfu)

D



---	---	---	---	+	+	+	+	+	TNF-α (10ng/ml)
---	---	+	---	---	---	---	+	---	siRNA IKKα (100nM)
---	---	---	+	---	---	---	---	+	DN-IKKβ (300pfu)
---	+	---	---	---	---	+	---	---	NT (100nM)
---	---	---	---	---	+	---	---	---	LacZ (300pfu)

Figure 3.15: The effect of Adv. DN-IKKβ and siRNA IKKα upon TNF-α-mediated p100 processing in HUVECs. Cells were transfected with non-targeting siRNA (100nM), siRNA IKKα (100nM) or infected with DN-IKKβ (300pfu) for 40h prior stimulation with TNF-α (10ng/ml) for 14h. Whole cell lysates were prepared, separated by SDS PAGE and assessed for A) p100 (100kDa), p52 (52kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) Fold stimulation of p52 and C) p100 are measured by scanning densitometry and D) The ratio of p100:p52, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments.

3.5 The role of IKKα in the regulation of NFκB transcriptional activity

The previous section demonstrated that IKKα is not involved in the up-stream regulation of the canonical NFκB pathway; however, there are numerous studies have demonstrated IKKα regulation of p65 dependent genes including ICAM-1, VCAM-1 and IL-8 as discussed in chapter 1. Therefore there is a possibility that IKKα plays a role in the regulation of NFκB (p65) transcriptional activity. In the following experiments, a luciferase assay was used and cells were infected with an Adv. NFκB-Luc gene expressing 5 repeat sequences (TGGGGACTTTCCGC) which represents a p65 dependent binding site.

3.5.1 TNF-α and IL-1β induced NFκB reporter activity in HUVECs

Figure 3.16 shows a time course of TNF-α (panel A) or IL-1β (panel B) induced NFκB reporter activity over 24h. HUVECs were infected with Adv. NFκB-Luc, Adv. DN-IKKα and/or β for 40h prior to simulation with 1ng/ml of TNF-α for the indicated time points. In panel A, the onset of the TNF-α induced NFκB reporter activity was very slow; an increase in activity was only observed after 4h stimulation with TNF-α. A maximum response was obtained by 6h to 8h (Fold stimulation = 31264 and 28989) and levels returned toward basal values after 24h stimulation. A similar pattern was observed for IL-1β, the onset of the response was found to be very slow and an increase of NFκB reporter activity was observed after 4h stimulation. A maximum response was obtained by 6h to 8h (Fold stimulation = 26307 and 28310) and again returned to the basal level after 24h stimulation. In addition, luciferase buffer alone did not elicit the NFκB luciferase activity without the presence of the agonist and Adv. NFκB-Luc. Adv. LacZ was used as negative control which had no effect on the response.

3.5.2 Characterisation of the role of IKKα in the regulation of TNF-α induced NFκB reporter activity

In figure 3.17, HUVECs were infected with Adv. NFκB-Luc, Adv. DN-IKKα and/or β for 40h prior to simulation with 1ng/ml of TNF-α for a further 8h. TNF-α alone dramatically increased the NFκB reporter activity (28920 fold). Although lower concentrations of Adv. DN-IKKα did not affect the response, the maximum concentration (300pfu) of Adv. DN-IKKα significantly reduced the NFκB reporter by 45%. As expected, 200pfu of Adv. DN-IKKβ was able to reduce approximately 40% of the reporter activity (% maximum

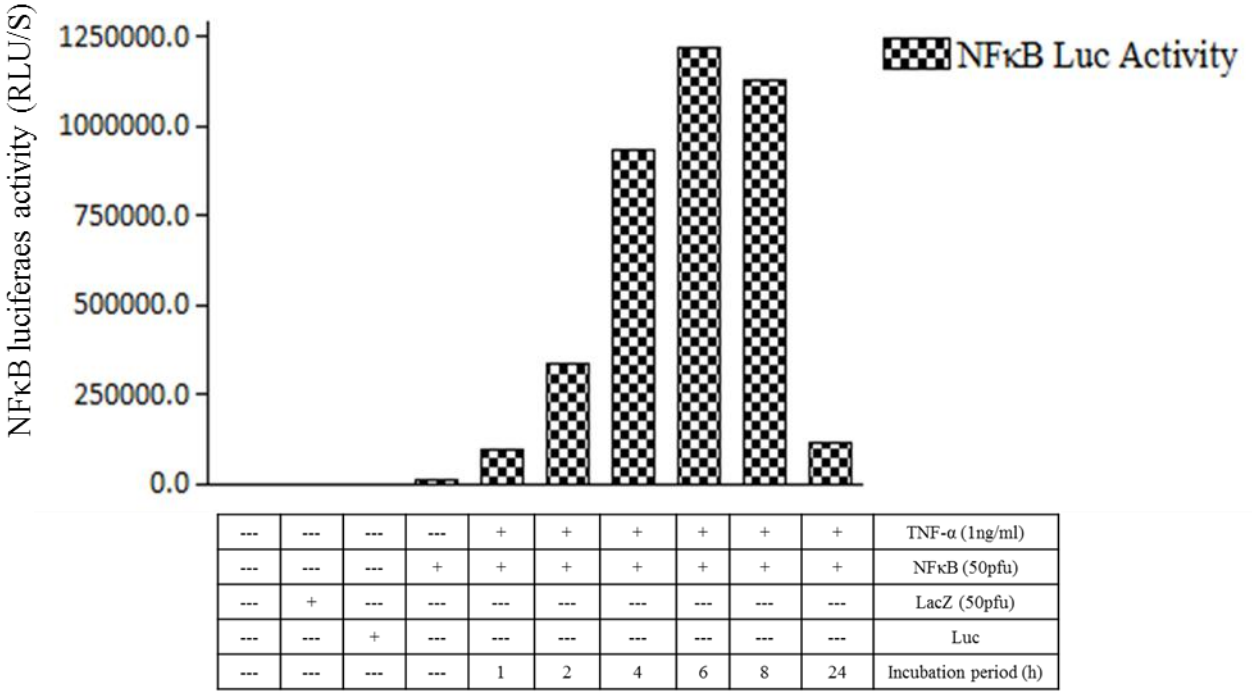
stimulation = 57.17 ± 20.22, n=3, p<0.01) and by approximately 70% in the presence of a maximum concentration (300pfu) of Adv. DN-IKKβ (% maximum stimulation = 30.14 ± 6.42, n=3, p<0.01). In order to determine the possibility of a synergistic or additive inhibitory effect, cells were infected with both Adv. DN-IKKα and β. A combination of a fixed concentrations. Adv. DN-IKKβ (50pfu) with increasing concentration of Adv. DN-IKKα (100pfu-300pfu) elicited a greater inhibitory effect than single kinase inhibition alone. Furthermore all the double treatments showed significant reduction in the response. However the effects were found to be additive rather than synergistic. For example, Adv. DN-IKKβ (50pfu) alone reduced about 18% of the NFκB reporter activity and 100pfu of Adv. DN-IKKα alone caused around 31%, whilst a combination of 50pfu Adv. DN-IKKβ with 100pfu of Adv. DN-IKKα brought down about 40% of the response. Addition of both values equal to 40%, which is almost equal to the % inhibitory of double IKK inhibition. This was also found in other concentrations of the combinations of Adv. DN-IKKα and β, therefore the double inhibition seemed to induce additive inhibitory effects rather than synergic effect.

3.5.3 Characterisation of the role of IKKα in the regulation of IL-1β induced NFκB reporter activity

In figure 3.17 panel B, HUVECs were infected with Adv. NFκB-Luc, Adv. DN-IKKα and/or β for 40h prior to simulation with 1ng/ml of IL-1β for a further 8h. Similar to the result in panel A, the presence of a low concentration of Adv. DN-IKKα did not affect the response, whilst 200pfu (% maximum stimulation = 54.86 ± 8.25, n = 3, p<0.05) and a maximum concentration of 300pfu (% maximum stimulation = 57.71 ± 1.66, n = 3, p<0.05) reduced 42% of the NFκB reporter activity. Again, lower concentrations of Adv. DN-IKKβ significantly inhibited the response, as did a maximum concentration (300 pfu) which reduced level by as much as 70% (% maximum stimulation = 31.48 ± 0.81, n=3, p<0.01). A combination of fixed concentrations of DN-IKKβ (50pfu) or (100pfu) with increasing concentration of Adv. DN-IKKα (100pfu-300pfu) elicited greater inhibitory effect than single kinase inhibition and all these treatments showed a significantly reduction in the response. For example, Adv. DN-IKKβ (50pfu) alone induced only 3.6% inhibition and 100pfu of IKKα alone elicited about 8.1% inhibition. Combination of 50pfu of Adv. DN-IKKβ with 100pfu of Adv. DN-IKKα brought down about 37% of the response. In addition, 50pfu of Adv. DN-IKKβ alone reduced 3.6% and Adv. DN-IKKα (300pfu) reduced about 42.3 % of the response, whilst the combined treatment of 50pfu of Adv. DN-IKKβ with 300pfu of Adv.

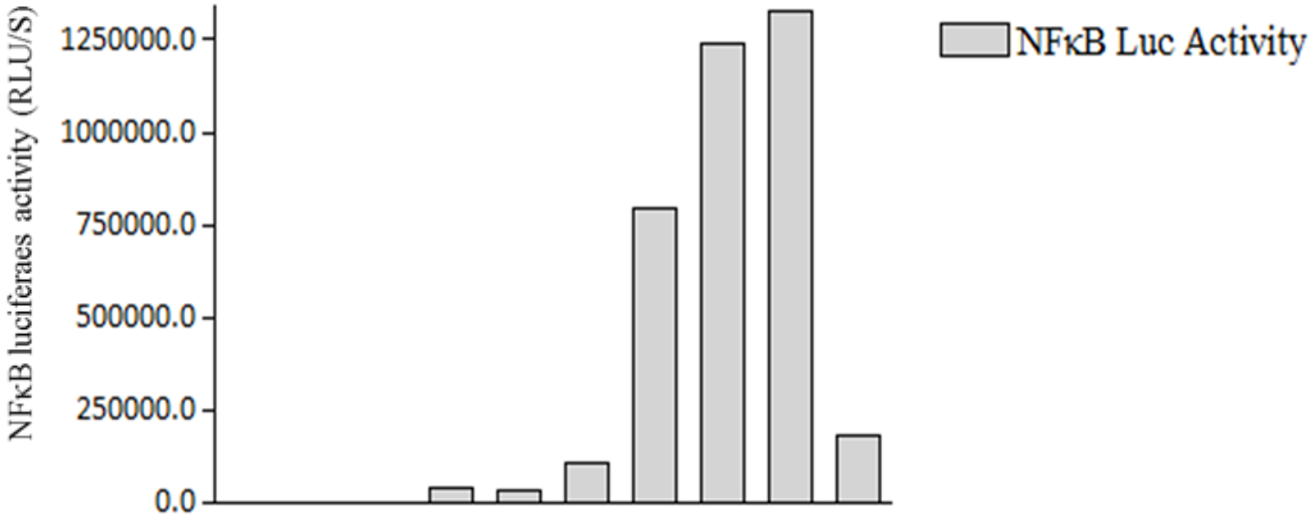
DN-IKKα resulted in 69% inhibition of the response, however addition of both values only equal to 46%, which is lower than the inhibitory effect of double inhibition, therefore there may be a synergic inhibitory effect on IL-1β induced NFκB reporter activity when blocking both IKKα and β. Moreover, combination of both Adv. DN-IKKα (100pfu)and β (100pfu) was able to induced maximum inhibitory effect, increasing the concentration of Adv. DN-IKKα to 100pfu of Adv. DN-IKKβ did not show a further reduction of the response.

A



---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)
---	---	---	+	+	+	+	+	+	+	NFκB (50pfu)
---	+	---	---	---	---	---	---	---	---	LacZ (50pfu)
---	---	+	---	---	---	---	---	---	---	Luc
---	---	---	---	1	2	4	6	8	24	Incubation period (h)

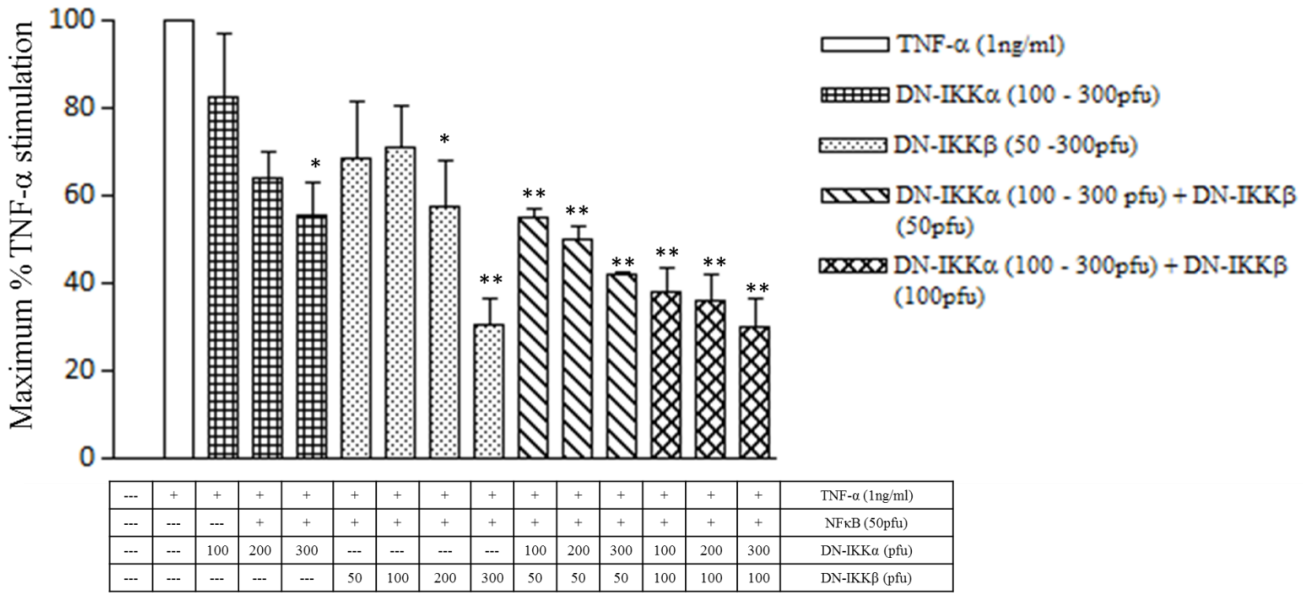
B



---	---	---	---	+	+	+	+	+	+	IL-1β (1ng/ml)
---	---	---	+	+	+	+	+	+	+	NFκB (50pfu)
---	+	---	---	---	---	---	---	---	---	LacZ (50pfu)
---	---	+	---	---	---	---	---	---	---	Luc
---	---	---	---	1	2	4	6	8	24	Incubation periods (h)

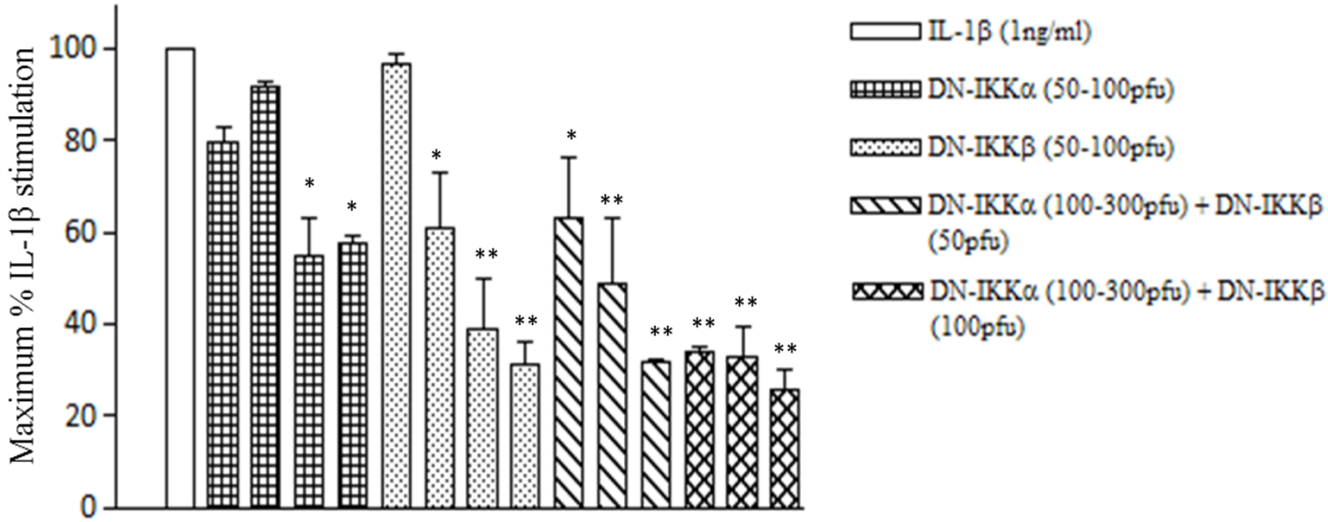
Figure 3.16: Time Course and concentration dependency of TNF-α or IL-1β mediated NFκB reporter activity in HUVECs. Cells were infected with Adv. NFκB-Luc 40h prior to the stimulation of A) TNF-α (1ng/ml) and B) IL-1β (1ng/ml). Cell lysates were then measured for luciferase activity as previously described in section 2.5. Data shown is expressed as NF-κB luciferase activity (RLU/s)

A



---	+	+	+	+	+	+	+	+	+	+	+	+	+	+	TNF-α (1ng/ml)
---	---	---	+	+	+	+	+	+	+	+	+	+	+	+	NFκB (50pfu)
---	---	100	200	300	---	---	---	---	100	200	300	100	200	300	DN-IKKα (pfu)
---	---	---	---	---	50	100	200	300	50	50	50	100	100	100	DN-IKKβ (pfu)

B



---	+	+	+	+	+	+	+	+	+	+	+	+	+	+	IL-1β (1ng/ml)
---	---	---	---	+	+	+	+	+	+	+	+	+	+	+	NFκB (50pfu)
---	---	50	100	200	300	---	---	---	---	100	200	300	100	200	300	DN-IKKα (pfu)
---	---	---	---	---	---	50	100	200	300	50	50	50	100	100	100	DN-IKKβ (pfu)

Figure 3.17: The effect of Adv. DN-IKKα on NFκB luciferase reporter activity mediated by TNF-α and IL-1β in HUVECs. Cells were infected with Adv. NFκB-Luc (50pfu) and additionally DN-IKKα (50-300pfu) and/or β (50-300pfu) as indicated for 40h prior to stimulation with A) TNF-α and B) IL-1β for 8h. Cell lysates were then measured for luciferase activity as previously described in section 2.5. Data shown is expressed as % agonist stimulation and each value represents the (mean ± s.e.m, n=3). *<0.05, **P<0.01 compared with agonist-stimulated control

3.6 The role of IKKα in the regulation of ICAM-1 and VCAM-1 expression in HUVECs

Although the previous study in our laboratory showed IKKα was not involved in the regulation of either ICAM-1 or VCAM-1 expression in human vascular smooth muscle cells (MacKenzie et al., 2007), the luciferase studies in this chapter showed the potential for IKKα to play a partial role in the regulation of the canonical NFκB pathway. Therefore, theoretically IKKα should be able to regulate p65 dependent genes. Indeed, there are numerous studies which have demonstrated that IKKα plays a role in the regulation of those p65 dependent genes (discussed in chapter 1). The following experiment was conducted to identify the role of IKKα in the regulation of TNF-α and IL-1β stimulated ICAM-1 and VCAM-1 expression.

3.6.1 TNF-α induced ICAM-1 and VCAM-1 expression in HUVECs in concentration dependent manner

It has been shown previously in the laboratory that TNF-α induced ICAM-1 and VCAM-1 in both HUVECs and human vascular smooth muscle cells (Al-Mutairi et al., 2010; MacKenzie et al., 2007). Therefore the aim of this initial experiment was to confirm the optimal concentration for TNF-α induced ICAM-1 and VCAM-1 expression. In figure 3.18, HUVECs were stimulated with increasing concentrations of TNF-α for 8h. The response to TNF-α was very sensitive, the lowest concentration (0.1ng/ml) was sufficient to strongly stimulate both ICAM-1 and VCAM-1 expression. Whilst both responses reached a maximum following stimulation with 10ng/ml of TNF-α, a standard concentration of 1ng/ml was used for subsequent studies.

3.6.2 Characterisation of the role of IKKβ in the regulation of TNF-α induced ICAM-1 and VCAM-1 expression in HUVECs.

It has been shown that IKKβ plays a key role in the regulation of both ICAM-1 and VCAM-1 expression (Denk et al., 2001; Mackenzie et al., 2007; Meiler et al., 2002). Figure 3.19 demonstrates a concentration curve for the effect of Adv. DN-IKKβ on TNF-α induced ICAM-1 and VCAM-1 expression in HUVECs. As expected, TNF-α induced both ICAM-1 and VCAM-1 expression after 8h (Fold stim = 17.59 and 60.83). Whilst this response was not altered following infection with Adv. Lac, increasing the concentration of Adv. DN-IKKβ gradually inhibited TNF-α induced ICAM-1and VCAM-1 expression in HUVECs in a

concentration-dependent manner. A maximum concentration of DN-IKKβ almost abolished both ICAM-1 and VCAM-1 expression (% inhibition = 74% and 76.3% respectively).

3.6.3 Characterisation of the role of IKKα in the regulation of TNF-α induced ICAM-1 and VCAM-1 expression in HUVECs.

The following experiment was then performed to determine whether IKKα shares similar characteristics with IKKβ in the regulation of ICAM-1 and VCAM-1 expression in HUVECs. Figure 3.20 shows a concentration curve for the effect of Adv. DN-IKKα on TNF-α induced ICAM-1 and VCAM-1 expression. TNF-α induced both ICAM-1 and VCAM-1 expression after 8h, increasing concentration of Adv. DN-IKKα gradually reduced ICAM-1 expression which was approximately 50% at a maximum of 300 pfu. In contrast low concentrations of DN-IKKα had little effect on VCAM-1 expression, although a maximum concentration (300pfu) gave a small but significant reduction of the VCAM-1 expression (% inhibition = 27.5%). The presence of the Adv. lacZ slightly reduced TNF-α induced ICAM-1 expression but did not alter VCAM-1 expression.

In order to confirm the results obtained with individual treatments, HUVECs were simultaneously pre-treated with a maximum concentration of Adv. DN-IKKα (300pfu) and Adv. DN-IKKβ (200pfu). In figure 3.21, ICAM-1 expression was found to be significantly increased following 8h stimulation with TNF-α (Fold stim = 20.06 ± 1.91, n = 3, $p<0.01$), whilst Adv. DN-IKKα reduced the response by 25 % (Fold stim = 13.7 ± 2.28, n = 3, $p<0.05$). As expected, Adv. DN-IKKβ caused more than a 75% reduction of the response (Fold stim = 2.87 ± 0.78, n = 3, $p<0.01$). Combined infection with both Adv. DN-IKKα and β completely abolished the expression of ICAM-1 (Fold stim = 0.78 ± 0.38, n = 3, $p<0.01$).

A similar result was obtained for TNF-α induced VCAM-1 expression (Figure 3.22). Inhibition of IKKα function by Adv. DN-IKKα only reduced the response by around 20% (Fold stim = 40.20 ± 4.43, n = 3, $p<0.05$). In contrast, the response was reduced by up to 80% following Adv. DN-IKKβ infection (Fold stim = 9.86 ± 4.72, n = 3, $p<0.01$). The additional presence of Adv. DN-IKKα enhanced the inhibitory effect of Adv. DN-IKKβ (Fold stim = 40.20 ± 4.43, n = 3, $p<0.01$).

In order to confirm the effect of Adv. DN-IKKα on TNF-α induced ICAM-1 and VCAM-1 expression, siRNA IKKα was used in the following experiment. In figure 3.23, HUVECs were pre-treated with either Adv. DN-IKKα, β or siRNA IKKα for 40h prior to stimulation of TNF-α. Alone 300pfu of DN-IKKα strongly inhibited TNF-α induced ICAM-1 expression, over 85% inhibition was observed. A low concentration of Adv. DN-IKKβ (75pfu) reduced ICAM-1 expression by approximately 30%. Interestingly, knockdown of endogenous IKKα using siRNA proved more effective and reduced ICAM-1 expression by more than 60%. Similar to the previous experiment, combining both DN-IKKα and β generated a greater inhibitory effect than blocking either of the kinases alone (% inhibition = 88.5%), IKKα knockdown and DN-IKKβ also induced a stronger inhibitory effect (% inhibitory = 80.6%). The non-targeting scrambled DNA had a slight inhibitory effect on the TNF-α induced response.

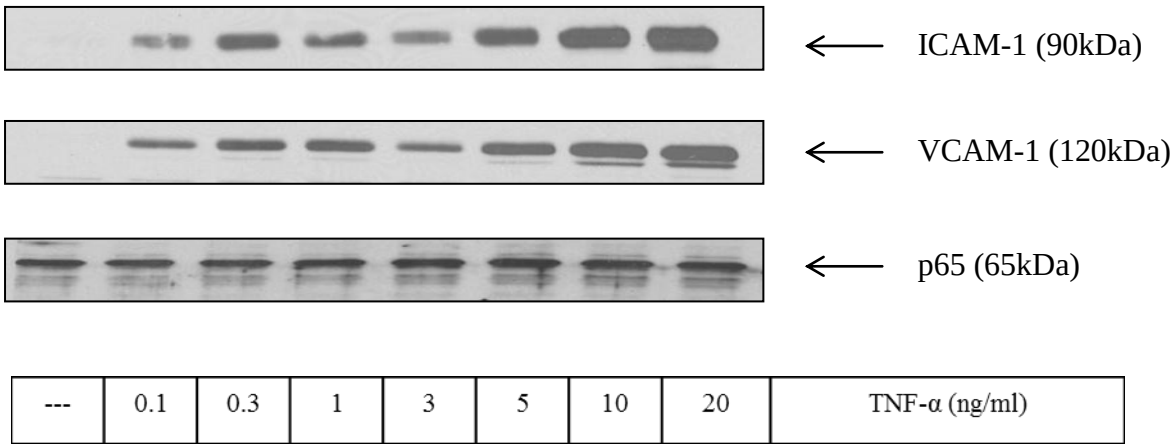
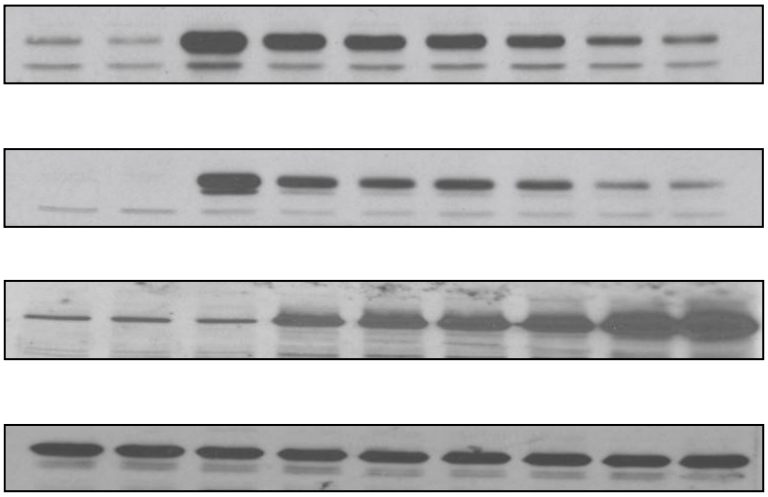
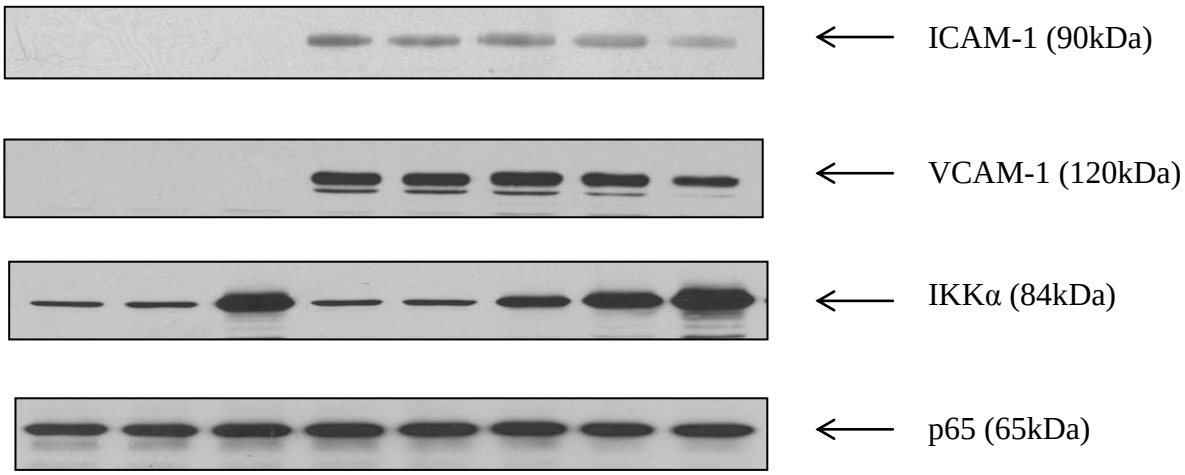


Figure 3.18: Concentration dependency of TNF-α-stimulated induction of ICAM-1 and VCAM-1 expression in HUVECs. Cells were stimulated with increasing concentration of TNF-α for 8h. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for ICAM-1 (90kDa), VCAM-1 (120kDa) and p65 (65kDa) expression as outlined in section 2.3.



---	---	+	+	+	+	+	+	+	TNF-α (1ng/ml)
---	---	---	50	75	100	125	150	200	DN-IKKβ (pfu)
---	+	---	---	---	---	---	---	---	LacZ (pfu)

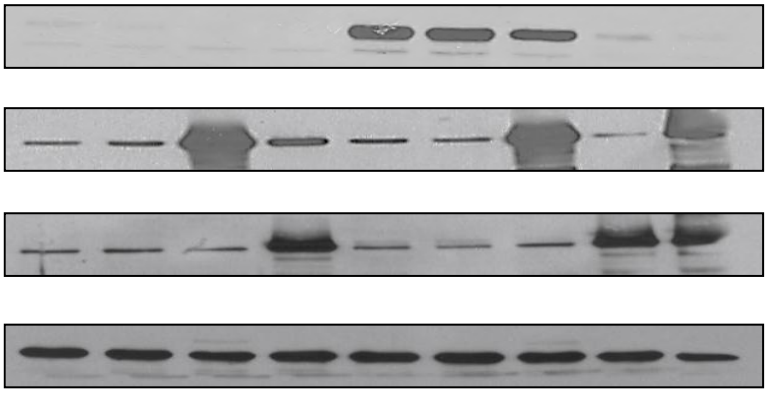
Figure 3.19: The effect of Adv. DN-IKKβ upon TNF-α-mediated ICAM-1 and VCAM-1 expression in HUVECs. Cells were infected with Adv. DN-IKKβ or LacZ for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for ICAM-1 (90kDa), VCAM-1 (120kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3.



---	---	+	+	+	+	+	+	TNF-α (1ng/ml)
---	---	300	---	---	100	200	300	DN-IKKα (pfu)
---	+	---	---	+	---	---	---	LacZ (pfu)

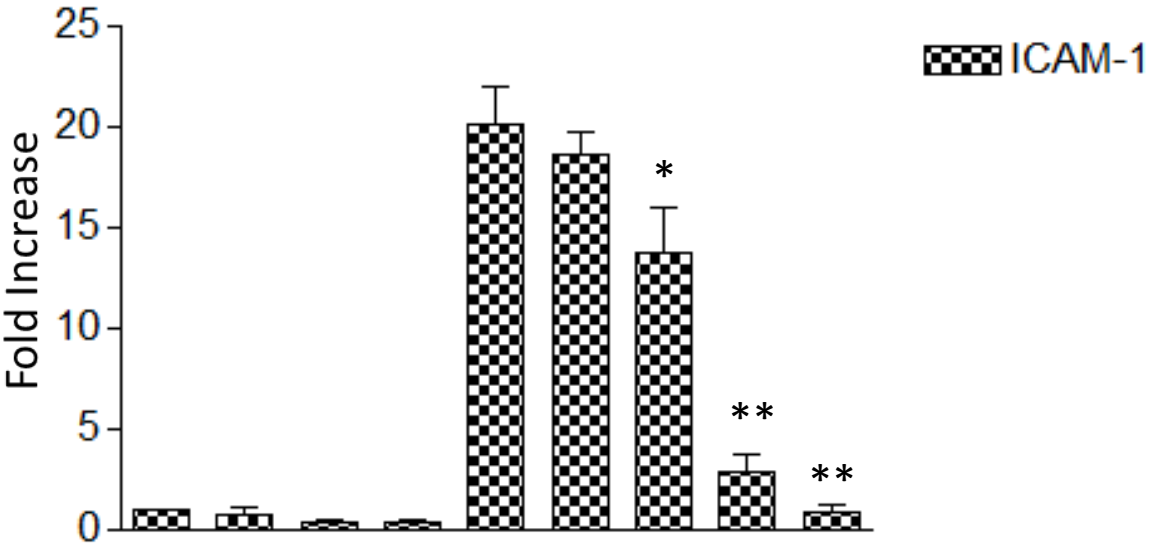
Figure 3.20: The effect of Adv. DN-IKKα upon TNF-α-mediated ICAM-1 and VCAM-1 expression in HUVECs. Cells were infected with Adv. DN-IKKα, or LacZ for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for ICAM-1 (90kDa), VCAM-1 (120kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3.

A



---	---	---	---	+	+	+	+	+	TNF-α (1ng/ml)
---	---	+	---	---	---	+	---	+	DN-IKKα (300pfu)
---	---	---	+	---	---	---	+	+	DN-IKKβ (200pfu)
---	+	---	---	---	+	---	---	---	LacZ (pfu)

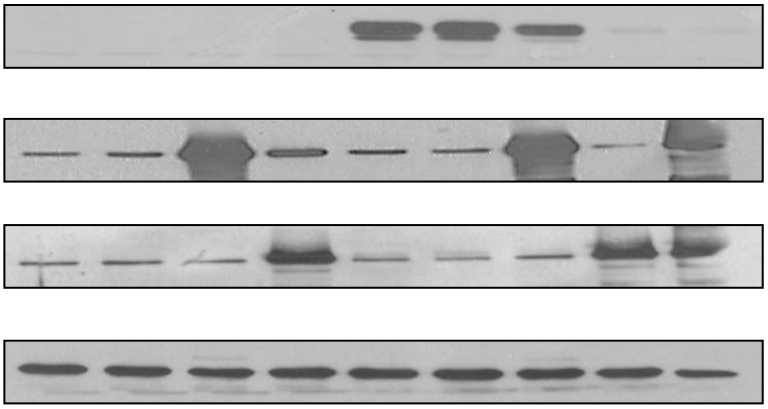
B



---	---	---	---	+	+	+	+	+	TNF-α (1ng/ml)
---	---	+	---	---	---	+	---	+	DN-IKKα (300pfu)
---	---	---	+	---	---	---	+	+	DN-IKKβ (200pfu)
---	+	---	---	---	+	---	---	---	LacZ (pfu)

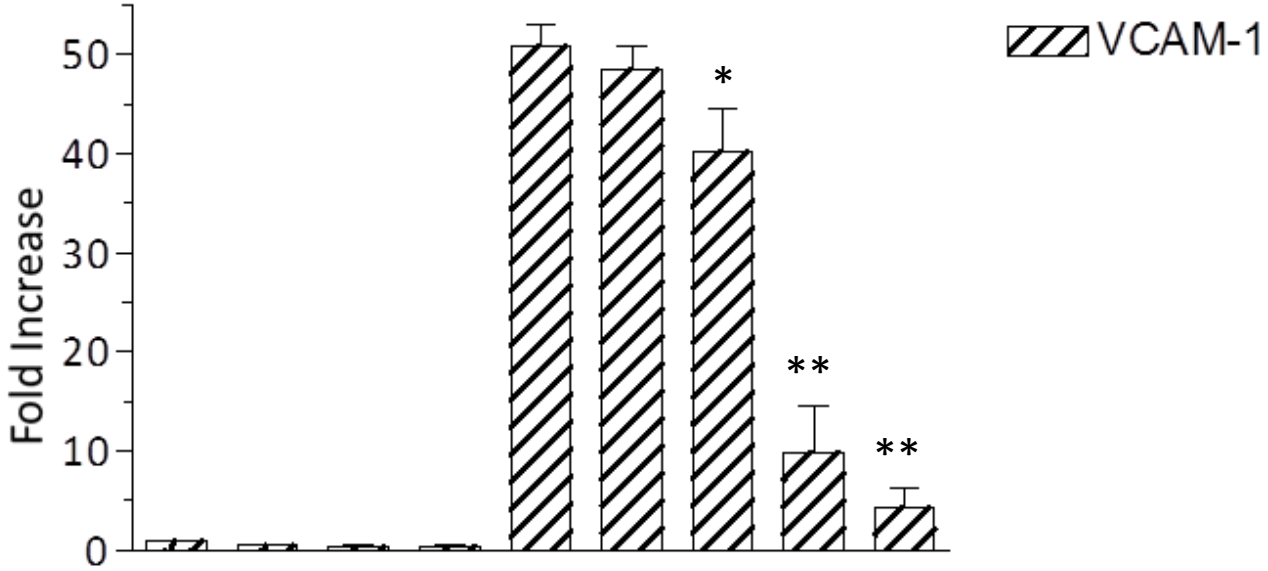
Figure 3.21 : The effect of Adv. DN-IKKα upon TNF-α -mediated ICAM-1 expression in HUVECs. Cells were infected with DN-IKKα, β or LacZ for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) ICAM-1 (90kDa), IKKα (84kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) Fold stimulation of ICAM-1 expression by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments. *P<0.05, **P<0.01 compared with agonist stimulated control.

A



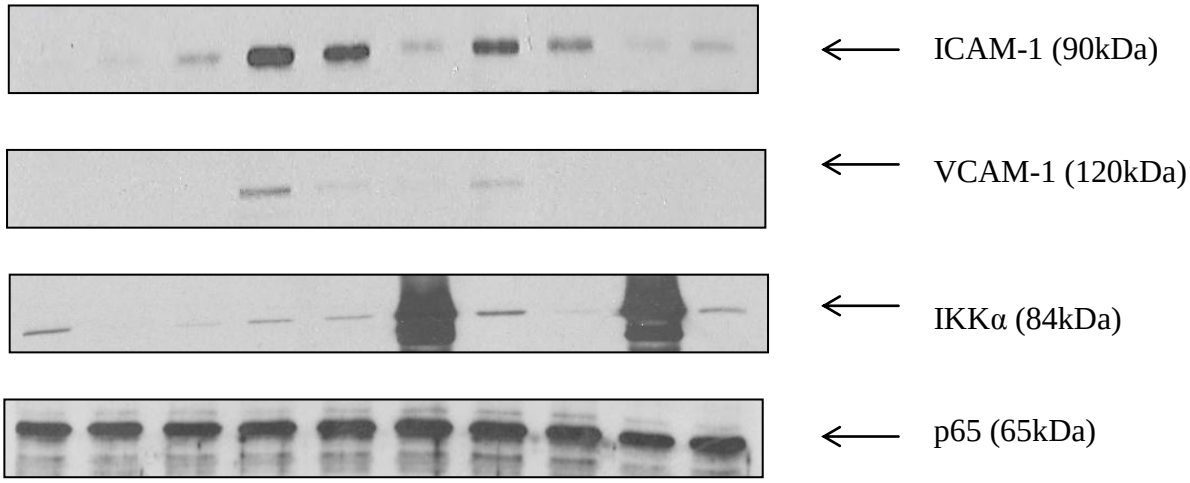
---	---	---	---	+	+	+	+	+	TNF-α (1ng/ml)
---	---	+	---	---	---	+	---	+	DN-IKKα (300pfu)
---	---	---	+	---	---	---	+	+	DN-IKKβ (200pfu)
---	+	---	---	---	+	---	---	---	LacZ (pfu)

B



---	---	---	---	+	+	+	+	+	TNF-α (1ng/ml)
---	---	+	---	---	---	+	---	+	DN-IKKα (300pfu)
---	---	---	+	---	---	---	+	+	DN-IKKβ (200pfu)
---	+	---	---	---	+	---	---	---	LacZ (pfu)

Figure 3.22: The effect of adv. DN-IKKα upon TNF-α -mediated VCAM-1 expression in HUVECs. Cells were infected with DN-IKKα, β or LacZ for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) VCAM-1 (120kDa), IKKα (84kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) Fold stimulation of VCAM-1 expression by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments. *P<0.05, **P<0.01 compared with agonist stimulated control.



---	---	---	+	+	+	+	+	+	+	TNF-α (1ng/ml)
---	---	+	---	---	+	--	---	+	---	DN-IKKα (300pfu)
---	+	---	---	---	---	---	+	---	+	siRNA IKKα (100nM)
---	---	---	---	---	---	+	---	+	+	DN-IKKβ (75pfu)
---	---	+	---	+	---	---	---	---	---	NT (100nM)

Figure 3.23: The effect of Adv. DN-IKKα and siRNA IKKα upon TNF-α - mediated both ICAM-1 and VCAM-1 expression in HUVECs. Cells were transfected with non-targeting scramble DNA, siRNA IKKα or infected with Adv. DN-IKKα or β for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for ICAM-1 (90kDa), VCAM-1 (120kDa), IKKα (84kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3.

3.6.4 Characterisation of the effect of IL-1 β on both ICAM-1 and VCAM-1 expression in HUVECs

Previous experiments conducted using TNF-α were repeated using IL-1β. Figure 3.24, panels A and B show time courses of ICAM-1 and VCAM-1 expression and concentration dependent responses induced by IL-1β. Following stimulation of HUVECs with 10ng/ml of IL-1β, a very slow lag time of 4 to 6h was observed before an increase in ICAM-1 expression was manifest. A maximum response was obtained between 8h and 12h of stimulation (Fold stim 25.18 and 29.58 respectively) but dropped to 13.61 fold by 24h. Similarly, VCAM-1 expression also showed a lag period of 4 to 6h before an increase in VCAM-1 expression was observed. Again maximum expression was achieved by 8h (Fold stim = 20.08), the response dropped to around 10 fold by 12h and returned back toward basal levels by 24h. In panel B, HUVECs were stimulated with increasing concentrations of IL-1β by 8h. Both ICAM-1 and VCAM-1 expression was not induced by very low concentrations of IL-1β (0.1-0.5ng/ml), however, significant expression was observed by 1ng/ml of the cytokine and reached a maximum level by 10ng/ml (Fold stim = 56.84 and 72.56 respectively).

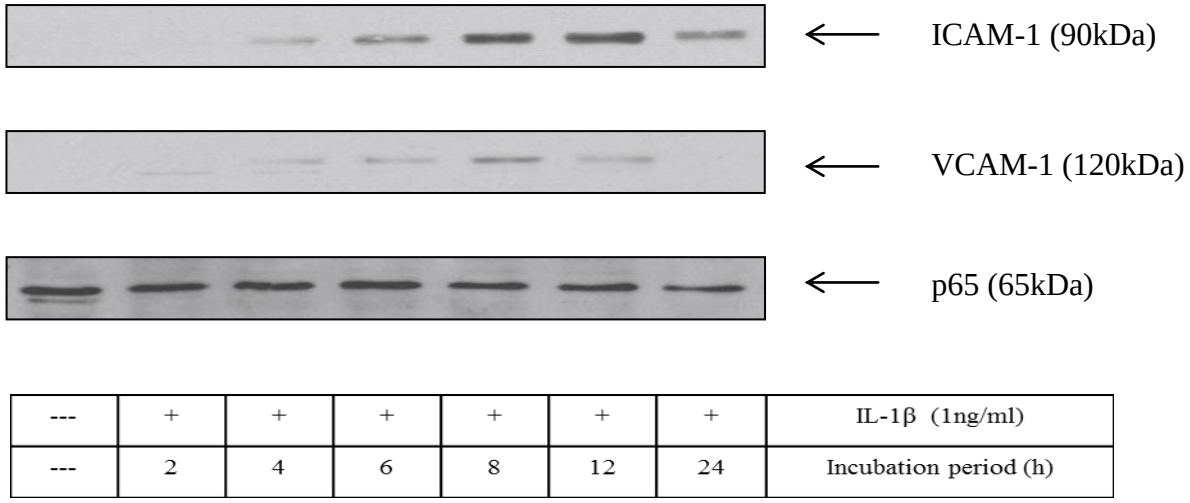
3.6.5 Characterisation of the role of IKKβ on IL-1 β induced both ICAM-1 and VCAM-1 expression in HUVECs

Figure 3.25 shows a concentration curve for the effect of Adv. DN-IKKβ on TNF-α induced ICAM-1 and VCAM-1 expression in HUVECs. HUVECs were infected with increasing concentrations of Adv. DN-IKKβ 40h prior to stimulation. As expected, IL-1β induced ICAM-1 expression after 8h (Fold stim = 32.66), increasing the concentration of Adv. DN-IKKβ gradually inhibited IL-1β induced ICAM-1 expression in HUVECs. A maximum concentration (200pfu) of Adv. DN-IKKβ almost abolished ICAM-1 expression (Fold stim = 4.43 and % maximum inhibition = 86.4%). The effect of DN-IKKβ on IL-1β induced VCAM-1 expression was also shown to be concentration dependent. IL-1β induced VCAM-1 expression after 8h (Fold stim = 22.79), a maximum concentration of DN-IKKβ (200pfu) again nearly abolished the expression of VCAM-1 (Fold stim = 2.79, % maximum inhibition = 87.7%).

3.6.6 Characterisation of the role of IKKα on IL-1 β induced ICAM-1 and VCAM-1 expression in HUVECs

In order to confirm the results obtained with individual treatments, HUVECs were simultaneously pre-treated with a maximum concentration of Adv. DN-IKKα (300pfu) and the lower Adv. DN-IKKβ (75pfu). In figure 3.26, ICAM-1 expression was found to be significantly increased following 8h stimulation with IL-1β (Fold stim = 26.96). Whilst Adv. DN-IKKα reduced by approximately 50% the ICAM-1 response (Fold stim = 13.53), 75pfu of Adv. DN-IKKβ reduced the ICAM-1 levels by 40% (Fold stim = 59.77). The double inhibition of both IKKα and β completely abolished the expression of ICAM-1 (Fold stim = 1.07). A similar result was obtained for VCAM-1 expression. A maximum concentration (300pfu) of DN-IKKα caused a 88% reduction in the expression of VCAM-1 whilst 75pfu of DN-IKKβ caused a 75% inhibition. Unsurprisingly, infection of both Adv. DN-IKKα and β resulted in virtual inhibition of VCAM-1 expression. (Fold stim = 0.14).

A



B

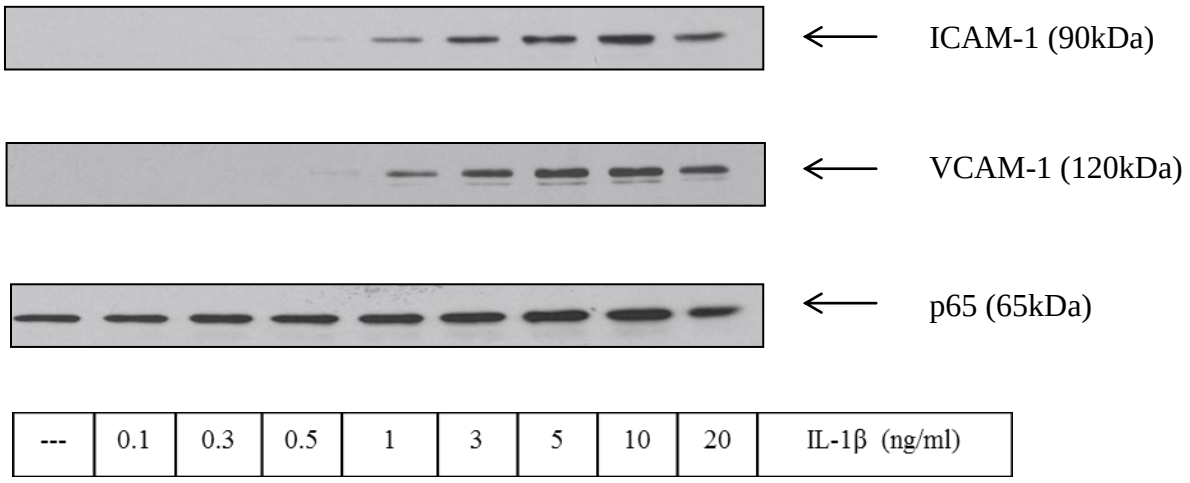
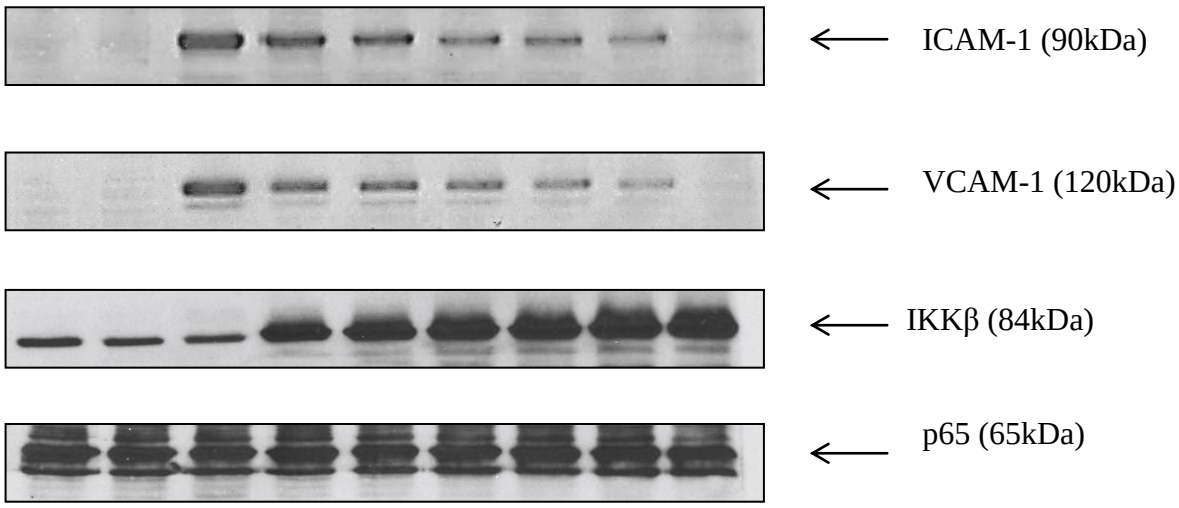
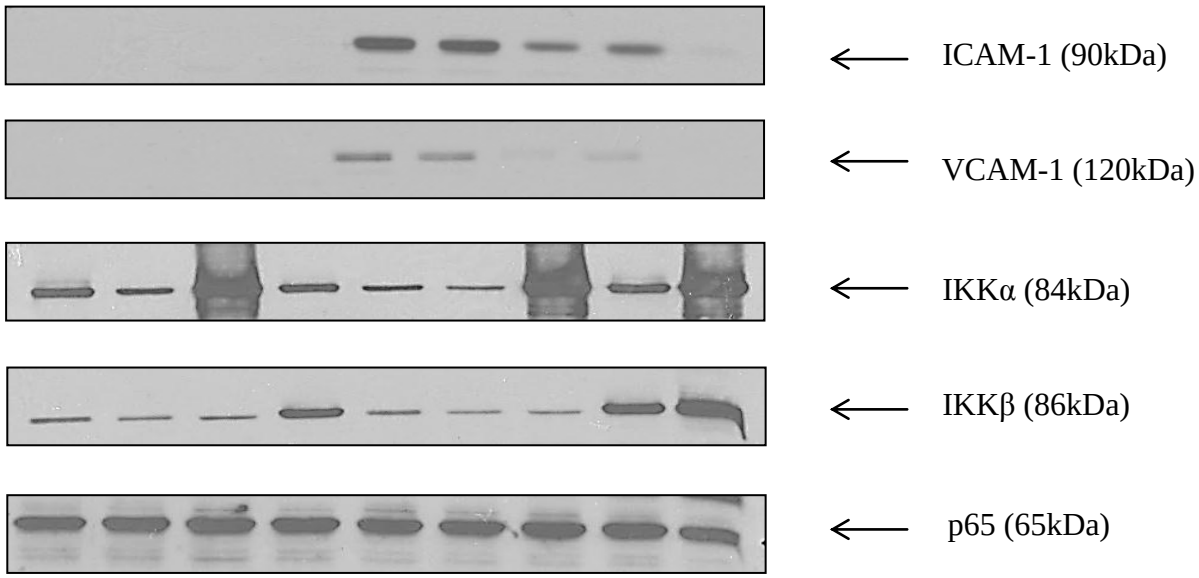


Figure 3.24: Time course and concentration dependency of IL-1β mediated ICAM-1 and VCAM-1 expression in HUVECs. A) Cells were stimulated with IL-1β (1ng/ml) for the indicated time points. B) Cells were stimulated with increasing concentrations of IL-1β for 8h. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for ICAM-1 (90kDa), VCAM-1 (120kDa), and p65 (65kDa) as outlined in section 2.3.



---	---	+	+	+	+	+	+	+	IL-1β (1ng/ml)
---	---	---	50	75	100	125	150	200	DN-IKKβ (pfu)
---	+	---	---	---	---	---	---	---	Laz (pfu)

Figure 3.25: The effect of Adv. DN-IKKβ upon IL-1β-mediated ICAM-1 and VCAM-1 expression in HUVECs. Cells were infected with Adv. DN-IKKβ or LacZ (300pfu) for 40h prior to stimulation with IL-1β (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for ICAM-1 (90kDa), VCAM-1 (120kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3.



---	---	---	---	+	+	+	+	+	IL-1β (1ng/ml)
---	---	+	---	---	---	+	---	+	DN-IKKα (300pfu)
---	---	---	+	---	---	---	+	+	DN-IKKβ (75pfu)
---	+	---	---	---	+	---	---	---	Laz (pfu)

Figure 3.26: The effect of Adv. DN-IKKα upon IL-1β –mediated ICAM-1 and VCAM-1 expression in HUVECs. Cells were infected with Adv. DN-IKKα, β or LacZ (300pfu) for 40h prior to stimulation with IL-1β (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for ICAM-1 (90kDa), VCAM-1 (120kDa), IKKα (84kDa), IKKβ (86kDa) and p65 (65kDa) as outlined in section 2.3.

3.7 Discussion

The aim of the chapter was to characterise the role of IKKα in both the canonical and non-canonical NFκB pathways in HUVECs. Whilst the significance of IKKβ in this cell type has been explored to some extent (Bu et al., 2005, De Martin et al., 2000), the role of IKKα has been largely neglected. Two approaches were considered, firstly the activation of the kinase itself and pathways known to be activated downstream and secondly, molecular inhibition of IKKα activity by using Adv. DN-IKKα and siRNA. These strategies have revealed an important role for IKKα in HeLa, MEFs and have implicated roles in cancer and cardiovascular disease.

The first section of this chapter sought to identify the ability of IKKα to undergo nuclear translocation in response to cellular stimulation. Figure 3.1 demonstrated that TNF-α induced IKKα nuclear translocation by 30 min, but the IKKα level returned to basal within 2 hours. Very few studies have examined IKKα nuclear translocation, however in comparison, Anest and co-workers demonstrated sustained TNF-α induced IKKα nuclear translocation in MEFs which lasted for as long as 120 min (Anest et al., 2003) and Yamamoto and co-workers showed that IKKα interacts with CBP following 30min of TNF-α stimulation in MEFs (Yamamoto et al., 2003). The duration of IKKα retention inside the nucleus may vary according to different cell types. IKKα is translocated to the nucleus due to expression of NLS within the kinase domain at the N-terminus (Gloire et al., 2007, Sil et al., 2004). However other components such as NFκB and recently IKKβ have also been shown to shuffle between the nucleus and the cytosol and this possibility could apply to IKKα (Accardi et al., 2011). Secondly, the duration of stimulation could be increased to determine if the action of IKKα is slow in onset or biphasic.

It was recognised that the studies using crude nuclear extraction were limited and attempts were made to utilise a more refined procedure using high speed centrifugation. However due to the lack of specific equipment, the improved protocol could not be applied routinely in the laboratory. Therefore immunofluorescence (direct staining) was used to confirm the result obtained using crude nuclear extracts. Due to non-specificity of the commercial IKKα antibody, cells were transfected with YFP-tagged IKKα DNA, and visualised using the microscopy. Again, figure 3.2A showed TNF-α induced IKKα transient nuclear translocation in the majority of the cell population by 30min. However, the transfection efficiency was

very poor with only around 20% of the whole population transfected with YFP-tagged IKKα DNA.

Due to the limitation of both techniques, the investigation of IKKα activation did not continue, however it would have been useful to determine if Adv. DN-IKKα affected nuclear translocation of IKKα. In fact, up to 700pfu of Adv. DN-IKKα did not affect nuclear translocation of IKKα (data not shown), this implies that the Adv. DN-IKKα (K44M) mutation inhibited ATP binding but not affect the affect the nuclear localisation sequence.

The second part of this chapter used molecular tools to study the role of IKKα in the regulation of NFκB pathways. Adv. DN-IKKα, siRNA IKKα and shRNA IKKα were used in the study. Firstly, DN-IKKα and also DN-IKKβ have been used routinely within the laboratory to define the role of IKKα and β in PAR-2 mediated NFκB signalling in keratinocytes (Goh et al., 2008) and in TNF-α stimulated human vascular smooth muscle cells, (MacKenzie et al., 2007). The Adv. DN-IKKα used in this study was mutated from serine to alanine within the ATP binding site (K44M) which inhibits the kinase activity. Several other studies have utilised this approach in a number of different cell types such as neuroblastoma (Armstrong et al., 2006), mouse melanoma cells (Rangaswami et al., 2006), rat aortic smooth muscle cells (Torrie et al., 2001) and colorectal cancer cells (Fernandez-Majada et al., 2007).

The advantage of using Adv. DN-IKKα is that in HUVECs the cytotoxic effect of a normal transfection method can be avoided. However, substantial overexpression of a dominant negative protein can lead to problems in specificity of inhibition. In other words, DN-IKKα may have off-target effects. Furthermore, there is evidence to suggest that IKKα can act as a scaffold protein in the absence of kinase activity (Li et al., 2002). In MEFs deficient in IKKα, reinsertion of DN-IKKα resulted in gain of function for the expression of a number of IKKα dependent genes. Therefore knockdown IKKα expression is necessary to confirm the effect of DN-IKKα in HUVECs.

Both siRNA and shRNA have been used widely to silence relevant target genes involved in a number of signalling pathways, however, siRNA and shRNA have distinct mechanisms to mediate target RNA interference pathway. Standard siRNA requires transfection reagents such as lipofectamine or polyethylenimine to access the cytoplasm. siRNA is loaded on to

RNA-interfering silencing complex (RISC) complex then undergoes the RNA interference process via targeting mRNA cleavage and degradation. Moreover, siRNA also binds Dicer and interacts with the double-stranded Tat RNA-binding protein (TRBP) or PACT (PKR activating protein) to mediate RNA interference and miRNA processing. Unlike siRNA, Adv. shRNA however does not require a transfection reagent to get inside the cell. The viral vector transports the shRNA into the nucleus for transcription. shRNA is transcribed by either RNA polymerase II and generates the primary transcript containing a hairpin like stem-loop structure. Then it is processed in the nucleus by a complex containing the RNase III enzyme Drosha and the double-stranded RNA-binding domain protein DGCR8. The processed primary transcripts is the pre-shRNA molecule. It is then transported to the cytoplasm by exportin 5. In the cytoplasm, the pre-shRNA is loaded onto the Dicer/TRBP/PACT complex where they are further processed to mature shRNA. Mature shRNA, then associates with argonaute protein containing RISC providing RNA interference function either through mRNA cleavage and degradation (Rao et al., 2009).

Adv. shRNA IKKα was used initially as the first option to knockdown IKKα because again adenoviral infection avoids stress or damage to the endothelial cell and the infection efficiency is routinely greater than 90%. Ironically, figure 3.3 showed that the maximum concentration (1000pfu) of Adv. shRNA IKKα failed to knockdown IKKα, whilst in contrast, the lowest concentration of siRNA IKKα (100nM) was sufficient to knockdown IKKα without causing a cytotoxic effect nor affecting endogenous IKKβ expression. It is generally accepted, as demonstrated by Rao and co-workers, that shRNA constructs are more suitable in utilising the endogenous processing machinery than siRNA constructs. Optimized shRNA constructs allow for a higher potency and sustainable effects resulting in less off-target effects (Rao et al., 2009). However, due to time constraints siRNA was used in subsequent experiments.

Having established what molecular tools would be suitable to inhibit IKKα activation their effects in HUVECs were examined. Firstly, the regulation of the canonical NFκB pathway was assessed. Agonists such as TNF-α and IL-1β are well known activators of the canonical NFκB pathway are therefore were used as stimulants. These cytokines are well recognised to activate the IKK complex leading to cellular IκB-α loss and p65 phosphorylation, subsequent p65 translocation into the nucleus and binding to a specific DNA sequence for transcription (discussed in chapter 1). Thus IκB-α loss and p65 phosphorylation were utilised as the key

markers of activation of the canonical NFκB pathway. As expected, Adv. DN-IKKβ reversed TNF-α induced IκB-α loss in HUVECs, a result consistent with a previous study in the laboratory which had demonstrated that IKKβ plays a key role in the regulation of NFκB pathway in HUVECs (Al-Mutairi et al., 2010). Actually, many studies have shown IKKβ to play a crucial role in the regulation of the canonical NFκB pathway. Tanaka and co-workers showed that TNF-α and IL-1β failed to induced IκB-α degradation in IKKβ deficient MEFs (Tanaka et al., 1999). Another study demonstrated that expression of DN-IKKβ (K44M) inhibited LPS induced NFκB activity in human monocytes and THP-1 monocytic cells (O'Connell et al., 1998). Furthermore, overexpression of kinase inactive IKKβ (K44A) in HeLa cells inhibited TNF-α induced nuclear translocation of RelA (Zandi et al., 1997). A similar response was observed in fibroblast-like synoviocytes, DN-IKKβ mutant prevented TNF-alpha-mediated nuclear translocation of NFκB (Aupperle et al., 1999).

In contrast, inhibition of IKKα by DN-IKKα did not affect the TNF-α induced IκB-α degradation in HUVECs (figure 3.6 and figure 3.7). Likewise, IKKα played no role in IκB-α loss in vascular smooth muscle cells (MacKenzie et al., 2007) and also human keratinocytes (Goh et al., 2008). In fact, a similar lack of inhibition was also observed in MEF from IKKα knockout mice (Solt et al., 2009). However, in contrast, Adli and co-workers demonstrated that TNF-α induced IκB-α loss was diminished in IKKα deficient MEFs and also in siRNA IKKα pre-treated HeLa cells (Adli et al., 2010). Furthermore, inhibition of IKKα blocked the IκB-α loss in HT-29 cells (DiDonato et al.,1997). A further study has revealed that IKKα can participate in the canonical pathway only in the absence of cellular IKKβ. These and other studies would suggest a cell type-specific participation of IKKα in degradation of IκB-α and subsequent nuclear translocation of p65.

Similarly, phosphorylation of p65 at serine 536 is also strongly associated with activation of the canonical NFκB pathway (discussed in Section 1.3.3.8). There are numerous studies which show blocking IKKβ activity resulted in inhibition of p65 phosphorylation at the serine 536 site (Goh et al., 2008; Yang et al., 2003). Furthermore Hall and co-workers demonstrated the overexpression of dominant-negative IKKα had no effect on p65 phosphorylation or TNF-alpha production, revealing that IKKβ, not IKKα, plays a central role in regulation of p65 phosphorylation at serine 536 and TNF-α production in the heart (Hall et al., 2005). As expected, figure 3.8 shows that TNF-α induced p65 phosphorylation at serine 536 was not

altered in the presence of DN-IKKα, whilst infection with Adv. DN-IKKβ resulted in a concentration dependent inhibition of the response.

Although TNF-α and IL-1β can both activate the canonical NFκB pathway through different up-stream signalling mechanism (discussed in chapter 1), Adv. DN-IKKα was also unable to affect IL-1β induced IκB-α loss and p65 phosphorylation. This confirms that IKKα is not involved in regulating the canonical NFκB pathway in a cytokine dependent manner. However, Solt and co-worker found that blocking IKKα resulted in inhibition of IL-1α induced p65 phosphorylation in MEFs. This may be due to IKKα playing a selective role in response to IL-1α stimulation (Solt et al., 2009). Although TNF-α, IL-1β and LPS are classified as the main agonists for activation of the canonical NFκB pathway, it has been discovered that IL-1α can also activate the canonical NFκB pathway in osteoclast-like cells (Jimi et al., 1996). IL-1α possesses higher affinity for IL-1 receptor type one (IL-1RI), whereas IL-1β displays higher affinity for IL-1 receptor type two (IL-1RII) (Ricote et al., 2004). Therefore IL-1α activated IL-1RI may result in phosphorylation and activation of IKKα which subsequently phosphorylates p65 in MEFs (Solt et al., 2009).

Although IKKα plays no role in the regulation of upstream intermediates of the canonical NFκB pathway, a number of studies have demonstrated that IKKα can influence NFκB transcriptional activity (Agarwal et al., 2005; Adli et al., 2010; Solt et al., 2009). However, the role of IKKα in this process in HUVECs remains uncertain. To answer this question, a NFκB luciferase assay was performed using an Adv. NFκB-Luc construct containing the consensus sequence 5'-GGGRNYYYCC-3', which has been identified as a specific p65/p50 binding site (Chen and Ghosh 1999, Chen et al 1998). As expected, inhibition of IKKβ dramatically reduced TNF-α and IL-1β induced p65 NFκB reporter activity, a high concentration of Adv. DN-IKKβ almost completely inhibited the response. Similar results have been obtained in other cell types, Yamamoto and co-workers showed that TNF-α induced NFκB luciferase activity was significantly diminished in IKKβ knockout MEF in comparison with wild type (Yamamoto et al., 2003). Also, DN-IKKβ (K44A) significantly inhibited Helicobacter pylori (H.pylori) induced NFκB luciferase activity in a human gastric cancer cell line (AGS) (Hirata et al., 2006). Studies also that showed that removal of endogenous IKKβ reduced TNF-α induced NFκB luciferase activity in HeLa and breast cancer cells (Adli et al., 2010). These studies overwhelmingly support a role for IKKβ

activity in mediating both NFκB translocation and serine 536 phosphorylation of p65 as a requisite for transcriptional activation.

In contrast, high concentrations of Adv. DN-IKKα gave only a partial inhibition of TNF- α or IL-1β induced NFκB luciferase activity. Similar results were obtained from other studies, inhibition of IKKα kinase activity resulted in reduction in NFκB luciferase activity in RKO colorectal cancer cells. A maximum concentration of DN-IKKα caused an approximate 85% inhibition of the response (Agarwal et al., 2005). Likewise, siRNA IKKα caused a marked reduction (around 50%) in TNF-α induced NFκB luciferase activity in HeLa and breast cancer cell (Adli et al., 2010). Moreover a knockout study showed that TNF-α induced NFκB luciferase activity was significantly decreased (around 86%) in IKKα deficient MEF in comparison to wild type and over 90% of IL-1α induced NFκB luciferase activity was reduced in IKKα knockout MEFs (Yamamoto et al., 2003). Taking these studies together. this may suggest IKKα seems to play a predominant role in the regulation of NFκB transcription activity in MEFs rather than in other cells. Inhibition of IKKα in HUVECs, HeLa and breast cancer cells resulted in around 40 to 50 % reduction of NFκB luciferase activity suggesting IKKα may not be as important in regulation of the canonical NFκB pathway in these cells types. If time had allowed, it would have been useful to use siRNA to confirm these observations.

If IKKα can regulate NFκB reporter activity mediated by the canonical pathway, it is possible that IKKα can subsequently regulate p65 dependent genes. In this chapter, the adhesion molecules ICAM-1 and VCAM-1 were selected as p65 dependent gene. Cytokines such as TNFα and IL-1β have been demonstrated to up-regulate both adhesion molecules in endothelial cells, a process known to be independent upon the NFκB pathway (Xia et al., 1998). Previous studies from our laboratory support a predominant role for IKKβ in the regulation of both ICAM-1 and VCAM-1 expression in either vascular smooth muscle cells (MacKenzie et al., 2007) or in HUVECs (Al-Mutairi et al., 2010). Indeed, infection with Adv. DN-IKKβ ablated ICAM-1 and VCAM-1 expression in response to TNFα or IL-1β (Figure 3.19). Moreover, Denk and co-workers also showed inhibiting IKKβ by retrovirus resulted in a decrease in the expression of both adhesion molecules on the HUVECs cell surface assessed by flow cytometry analysis (Denk et al., 2001). Furthermore, Meiler and co-workers also showed Adv. DN-IKKβ supressed TNF-α induced ICAM-1 and VCAM-1

expression in HUVECs (Meiler et al., 2002). A recent study utilised 7-Hydroxyfrullanolide (7HF), a sesquiterpene lactone which was to found inhibit nuclear translocation of NFκB by directly inhibiting the phosphorylation of IKKβ. This compound leads to suppression of ICAM-1, VCAM-1 and E-selectin expression in LPS-stimulated endothelial cells (Fonseca et al., 2011). These and other approaches confirm that IKKβ plays a crucial role in the regulation of the expression of adhesion molecules in HUVECs.

As the previous results showed IKKα to partially regulate NFκB (p65) transcriptional activity, it is possible that IKKα may also be able to regulate ICAM-1 and VCAM-1 expression. Indeed, infection with a maximum concentration of Adv. DN-IKKα (300pfu) resulted in a significant reduction in TNF-α induced ICAM-1 and VCAM-1 expression. By comparison with Adv. DN-IKKβ, the inhibitory effect of Adv. DN-IKKα was weaker and only caused a partial inhibition (figure 3.21 and 3.22). Interestingly, even using up to 1000pfu of DN-IKKα did not affect the TNF-α induced ICAM-1 and VCAM-1 expression in human vascular smooth muscle cells (MacKenzie et al., 2007) nor altered the enterovirus 71 (EV71) induced VCAM-1 expression in rat vascular smooth muscle (Tung et al., 2007), suggesting that the role of IKKα in the regulation of both ICAM-1 and VCAM-1 is again cell type dependent. Studies in this chapter however also confirmed a role of IKKα in the regulation of adhesion molecule expression in endothelial cell and the majority of recent studies support this finding. For example, inhibiting IKKα kinase activity using retroviral infection with DN-IKKα resulted in complete inhibition of TNF-α induced VCAM-1 and IL-8 and partially reduced ICAM-1, MCP-1 and E-selectin expression on the HUVECs cell surface (Denk et al., 2001). In addition, Gloire and co-workers showed mutation of the NLS resulted in inhibition of the TNF-α induced ICAM-1 expression (Gloire et al., 2007). Also, mutation of IKKα$^{AA/AA}$ (S176A, S180A) leads to inhibition of TNF-α induced ICAM-1 expression in mouse stromal cells (Bonizzi et al., 2004). Furthermore, Huang and co-workers demonstrated IKKα plays a role in the regulation of TNF-α induced ICAM-1 expression by directly phosphorylating CBP in the nucleus of HeLa cells (Huang et al., 2007). Similarly, DN-IKKα also inhibited TNF-α inducibility of the VCAM-1 promoter (Tu et al., 2001).

In chapter 1, evidence was discussed relating to a role for IKKα in the regulation of the non-canonical NFκB pathway. As described in this chapter, the ligands LTα1β2, BAFF and RANKL are the common agonists used to activate the pathway, however recently, it has been shown that TNF-α is also associated with the activation of non-canonical NFκB pathway

(Madge et al., 2008). Therefore TNF-α was tested as an activator of the non-canonical pathway in HUVECs. In these cells, TNF-α stimulated a time dependent increase in both p100 and p52 levels. The increase in p52 levels increased concomitantly with p100 expression in response to TNF-α, suggesting that p100 may dictate the levels of p52 irrespective of whether bonafide processing is on-going. Nevertheless, processing was not observed following IL-1 treatment suggesting a difference in the mechanism of activation by the two cytokines. Infection with Adv. DN-IKKα inhibited p100 processing and significantly reducing p52 levels without affecting p100 expression, this suggests that TNF-α is mediating p100 processing in a IKKα dependent manner. Likewise, knockdown of IKKα using siRNA also caused inhibition of p100 processing. Dejardin and co-workers showed p100 processing was inhibited in both DN-IKKα transfected MEFs and IKKα deficient MEFs (Dejardin et al., 2002). Furthermore, a recent study has confirmed that inhibiting IKKα leads to inhibition of p100 processing in HUVECs (Madge et al., 2008).

Surprisingly, infection of HUVECs with Adv. DN-IKKβ increased cellular expression of p52. A recent paper has demonstrated in both HUVECs and MEFs that blocking IKKβ resulted in the enhancement of the p52 levels and the expression of CXCL12, a protein dependent on the non-canonical pathway (Madge and May, 2010). This implies that the canonical NFκB pathway may negatively regulate the non-canonical pathway in HUVECs. Interestingly, blocking both IKKα and β using the non-selective IKK inhibitor BMS-345541 also led to inhibition of p100 processing but also protein expression. This again supports the notion that an increase in p52 levels may also be dependent on the relative expression of p100. To investigate this further the ratio of p52 formation vs p100 expression was examined. If TNF-α is only able to increase the expression of p100 rather than driving processing, the p100/p52 ratio would not be altered. Furthermore, following either DN-IKKα and siRNA IKKα treatment, the ratio would remain the same. However, the results were very difficult to interpret. As Adv. DN-IKKα clearly only reduced the p52 formation without affecting the p100 level, the level of p100/p52 ratio should increase, however the result did not show Adv. DN-IKKα and siRNA IKKα significantly increased the ratio of p100/p52. Both Adv. and siRNA IKKα showed an increase of the ratio but was not significantly different when compared with the agonist control. Taken together it is impossible to determine truly if p52 formation is dependency on p100 processing. Moreover TNF-α largely increased the expression of p100, and a small reduction of p52 level may not be sufficient to give a significantly increase of p100/p52 ratio.

To summarise the results presented in this chapter, IKKα was found not to be involved in the regulation of the canonical NFκB pathway directly, but partially regulated the p65 transcriptional activity and the expression of p65 dependent genes such as ICAM-1 and VCAM-1. These effects are consistent with earlier studies which demonstrated that following stimulation IKKα could translocate to the nucleus. A number of studies have shown that TNF-α induces IKKα nuclear translocation (Anest et al., 2003) and subsequent regulation of specific p65 dependent genes, such as IL-8, through the direct interaction with the promoter of the gene (Yamamoto et al., 2003). Moreover, Gloire and co-workers showed that whilst blocking IKKα activity did not affect the p65 translocation into the nucleus, p65 dependent gene expression however was inhibited (Gloire et al., 2007). Furthermore deletion of IKKα or mutation within the activation loop or NLS all lead to complete inhibition of TNF-α induced p65 binding to the ICAM-1 and MCP-1 promoters in MEFs (Gloire et al., 2007). In addition, Huang and co-workers found that IKKα mediated phosphorylation of the nuclear protein CBP, is crucial for p65 binding to genes promoter such as ICAM-1 and cIAP-2 in HeLa cells (Huang et al., 2007). These experiments support the findings presented in this chapter which demonstrated that IKKα plays a key role in the regulation of the non-canonical pathway in HUVECs. This implies that IKKα may regulate a number of non-canonical dependent genes such as CXCL12. Therefore inhibiting IKKα could be a potential target for novel therapeutic strategies aimed at blocking the altered endothelial cell phenotype at sites of chronic inflammation.

Chapter 4

Characterisation of the inhibitory effect of IKKα selective inhibitors (SU compounds) on Canonical and Non-Canonical NFκB pathways

4.1 Introduction

Experiments in the previous chapter established the potential for IKKα to play a role in the regulation of specific NFκB controlled events relevant in a condition such as atherosclerosis where endothelial cell dysfunction is a feature. However, there are very few studies which have examined the potential to utilise pharmacological inhibitors in a context of atherosclerosis. A single study has shown that the non-specific NFκB inhibitor PDTC attenuates atherosclerosis by selectively inhibiting transcription factor NFκB (Jawien et al., 2005). Early studies demonstrated that PDTC inhibits NFκB translocation into the nucleus (Ziegler-Heitbrock et al., 1993) and later Li and co-workers found that PDTC did not affect IKK phosphorylation of IκB-α but interfered with the ubiquitylation process and so prevented IKKα degradation (Li et al., 2006). Apart from proteasome inhibitors, there are also specific transcription factor NFκB inhibitors that inhibit NFκB translocation into the nucleus without affecting IκB-α degradation such as aromatic diamine compound NF-κB Activation Inhibitor II, JSH-23 (sc-222061) (Shin et al., 2004).

Whilst the compounds outlined above may not be ideal for inhibiting NFκB activity and hence inflammation, nevertheless, inhibition of IKKβ remains an attractive approach for diseases such as arthritis and cancer, so numerous IKKβ selective and non-selective IKK inhibitors have been developed. The IKKβ inhibitors widely studied include; PHA-408, PF-184 (Sommers et al., 2009), TPCA-1 (Birrell et al., 2005), PS-1145 (Castro et al., 2003) and ML120B (Schopf et al., 2006) and also non-selective IKK inhibitors such as BMS-345541 (Burke et al., 2003, MacMaster et al., 2003) and 4-Phenyl-7-azaindoles (Liddle et al., 2009). By comparison with IKKβ, no commercial IKKα specific inhibitors have been developed as yet but remain an attractive approach for cardiovascular inflammatory diseases. In collaboration with the medicinal chemistry group at the University of Strathclyde, a number of novel compounds were generated which *in vitro* studies indicated to be IKKα selective inhibitors. Therefore the aim of this chapter was to characterise the selectivity and the potency of these novel compounds (SU) as inhibitors of IKKα function in HUVECs. The inhibition of NFκB mediated pro-inflammatory molecules such as ICAM-1 and VCAM-1 could be a useful therapeutic approach and several studies have shown that pharmacological inhibition of IKKβ can prevent induction of these molecules. However, evidence clearly demonstrates that blocking p65 NFκB translocation or direct inhibition of IKKβ will necessarily result in endothelial cell apoptosis which will be detrimental to vessel integrity.

Therefore the ideal treatment would be to block both ICAM-1 and VCAM-1 whilst not affecting pro-survival gene expression. This could be achieved by selectively blocking IKKα.

4.2 Determining the dissociation constant (Ki value) of the SU compounds

The effects of two prototypic IKKα inhibitor compounds, SU1007 and SU1010 on IKKα/β kinase activities were examined using *in vitro* kinase assay. The kinase assay for this project was performed by Mrs. Louise C. Young using a kinase assay kit (Cell Signaling Technology, Inc., Danvers, MA, USA) with minor modifications. Both IKKα and IKKβ were purified to homogeneity and then the effect of SU compounds on either IKKα or IKKβ activity assessed The kinase reaction utilised a biotinylated peptide substrate containing the residues serine 32 of IκB-α. The extent of phosphorylation was assessed by dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) and the time-resolved fluorescence (TRF) for three individual experiments to determine the IC_{50} curves. In table 5.1, the Ki value (dissociation constant) of SU1007 for IKKα was 0.182μM ± 0.076μM, while the Ki value of IKKβ was about 100 fold higher than IKKα (Ki value= 22 μM ± 13 μM) Similarly, the Ki value for SU1010 on IKKα is very low (Ki value= 0.36 μM ± 0.15 μM), while the Ki value for IKKβ is about 83 fold higher than IKKα (Ki value= 30 μM). These data suggest that these compounds are relatively selective for IKKα.

A

SU1007

Ki value (μM)	Prism (n=3)	n=1	n=2	n=3	Ki (±SEM)
IKKα	0.093	0.058	0.17	0.32	0.182 (0.076)
IKKβ	10	30	6	30	22 (13)

B

SU1010

Ki value (μM)	Prism (n=3)	n=1	n=2	n=3	Ki (±SEM)
IKKα	0.31	0.26	0.16	0.66	0.36 (0.15)
IKKβ	30	30	30	>30	30 (?)

Table 4.1: The effects of SU1007 or SU1010 on IKKα and β kinases activities. IKKα and IKKβ were purified and the selectivity of SU compounds were assessed. The Ki value of SU1007 for IKKα is 0.093-0.32, whilst the Ki value for IKKβ is 10-30. The Ki value for IKKα is almost 100 folds higher than IKKβ. Similarly, the Ki value of SU1010 for IKKα is 0.31-0.66, whilst the Ki value for IKKβ is around 30. Again the Ki value for IKKα is almost 100 folds higher than IKKβ.

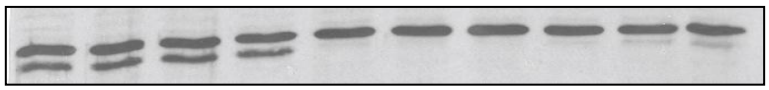
4.3 Characterisation of SU compounds on TNF-α or IL-1β induced activation of the NFκB canonical pathway in HUVECs

The previous chapter (figures 3.6 & 3.8) demonstrated that IKKα was not involved in the upstream regulation of canonical NFκB pathway. Therefore the SU compounds were tested for effects within the canonical pathway to determine if these inhibitors were selective in their actions within HUVECs.

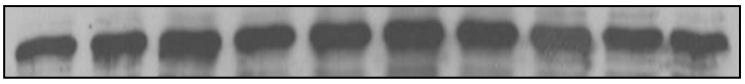
4.3.1 Characterisation of the effect of SU compounds on TNF-α induced IκB-α degradation in HUVECs

Initially the effect of both putative selective IKKα inhibitors on two markers of the canonical pathway signalling, cellular IκB-α loss or p65 phosphorylation was examined. Figure 4.1 shows that pre-treatment of HUVECs with SU1007 alone did not alter the basal level of IκB-α within the cells. As demonstrated previously, TNF-α induced a significant loss in cellular IκB-α, however, increasing the concentration of SU1007 did not result in a significant reversal. The equal cellular expression of p65 confirmed that the compound had no detrimental effect on cellular integrity. A similar result was obtained following pre-treatment with SU1010, the presence of compound did not affect either the basal level of IκB-α nor TNF-α induced IκB-α loss (figure 4.2).

A



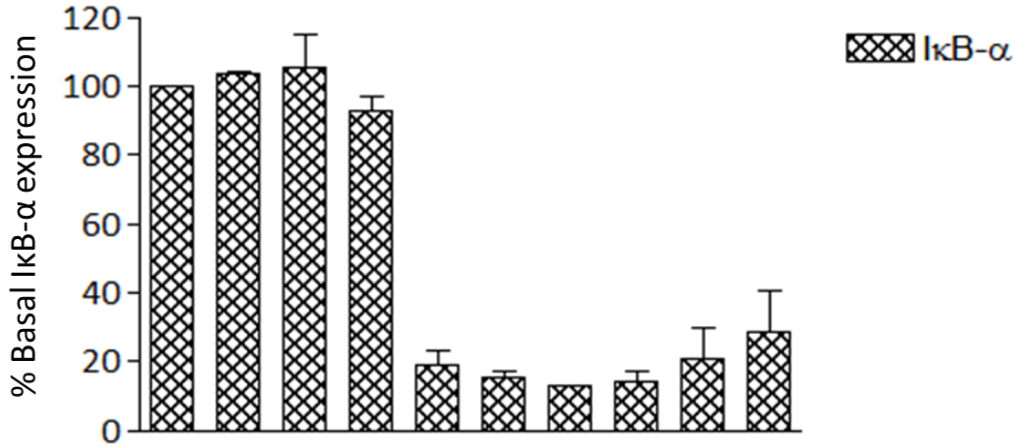
IκB-α (37kDa)



p65 (65kDa)

---	3	10	30	---	0.3	1	3	10	30	SU 1007 (μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

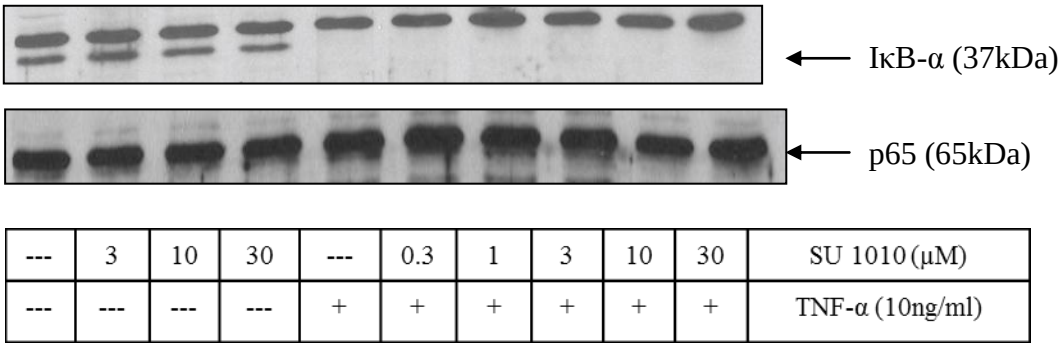
B



---	3	10	30	---	0.3	1	3	10	30	SU 1007 (μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

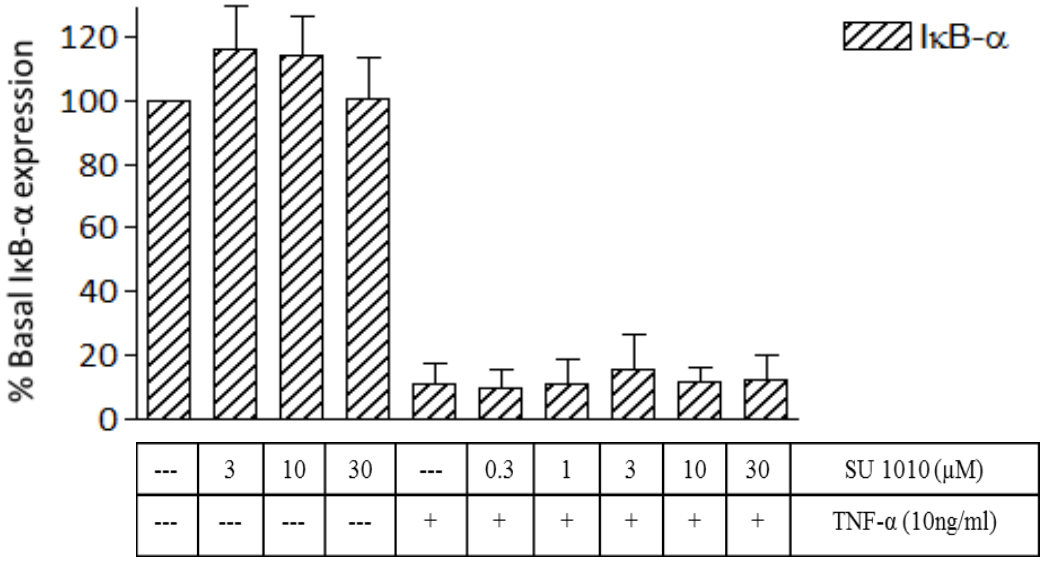
Figure 4.1: The effect of SU1007 upon TNF-α-mediated IκB-α degradation in HUVECs. Cells were pre-treated with SU1007 for 1h prior to the stimulation with TNF-α (10ng/ml) for 15min. Whole cell lysates were prepared, separated by SDS PAGE and assessed for A) IκB-α (37kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for b) % expression by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments.

A



---	3	10	30	---	0.3	1	3	10	30	SU 1010 (μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

B



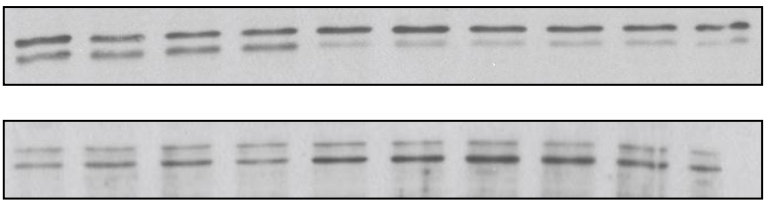
---	3	10	30	---	0.3	1	3	10	30	SU 1010 (μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

Figure 4.2: The effect of SU1010 upon TNF-α-mediated IκB-α degradation in HUVECs. Cells were pre-treated with SU1010 for 1h prior to the stimulation with TNF-α (10ng/ml) for 15min. Whole cell lysates were prepared, separated by SDS PAGE and assessed for A) IκB-α (37kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for b) % expression by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments.

In order to ensure that using 10ng/ml of TNF-α did not result in supramaximal stimulation, thereby masking any inhibition, the concentration was reduced 10 fold to 1ng/ml for cellular stimulation. Once again, figure 4.3, panel A demonstrates that this concentration whilst being sufficient to induce IκB-α loss, this was not altered by increasing concentrations of SU1007. A similar outcome was observed with SU1010 where low concentrations did not affect the response induced by TNF-α, whilst a maximum concentration (30μM) of SU1010 slightly reversed cellular IκB-α loss.

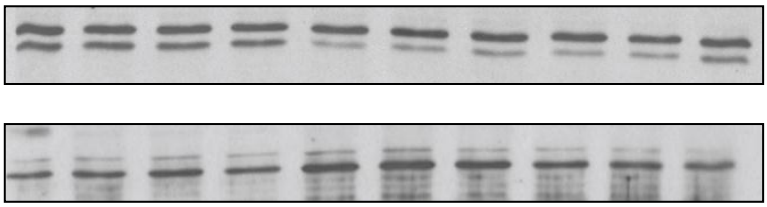
In order to determine if the initial incubation period of 1h was insufficient to demonstrate inhibition, HUVECs were pre-treated with SU compounds for 8h, prior to stimulation with TNF-α. Figure 4.4 shows that both SU 1007 and SU1010 did not alter TNF-α stimulated IκB-α loss even at a maximum concentration of 30μM. Moreover, neither IKKα nor p65 expression were affected by both compounds suggesting no detrimental effect on the cell.

A



---	3	10	30	---	0.3	1	3	10	30	SU 1007 (μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

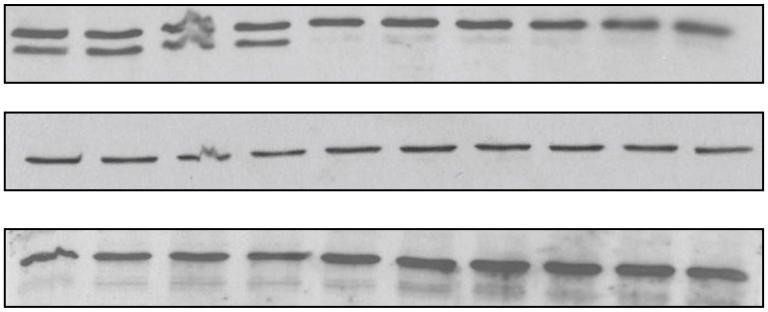
B



---	3	10	30	---	0.3	1	3	10	30	SU 1010 (μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

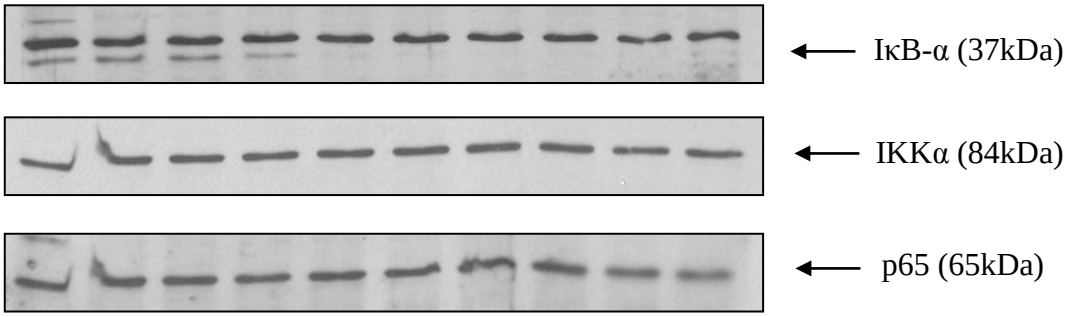
Figure 4.3: The effect of SU1007 and SU1010 upon TNF-α-mediated IκB-α degradation induced by 1ng/ml of TNF-α in HUVECs. Cells were pre-treated with A) SU1007 or B) SU1010 for 1h prior to the stimulation with TNF-α (1ng/ml) for 15 min. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for IκB-α (37kDa) and p65 (65kDa) as outlined in section 2.3. The result is representative of 1 experiment.

A



---	3	10	30	---	0.3	1	3	10	30	SU 1007 (μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

B



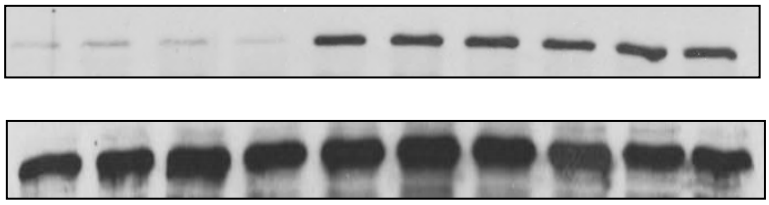
---	3	10	30	---	0.3	1	3	10	30	SU 1010 (μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

Figure 4.4: The effect of prolonged pre-treatment of SU1007 and SU1010 upon TNF-α-mediated IκB-α degradation in HUVECs. Cells were pre-treated with A) SU1007 or B) SU1010 for 8h prior to the stimulation with TNF-α (10ng/ml) for 15min. Whole cell lysates were prepared, separated by SDS PAGE and assessed for IκB-α (37kDa), IKKα (84kDa) and p65 (65kDa) as outlined in section 2.3. The result is representative of 1 experiment.

4.3.2 Characterisation of the effect of SU compounds on TNF-α induced p65 phosphorylation in HUVECs

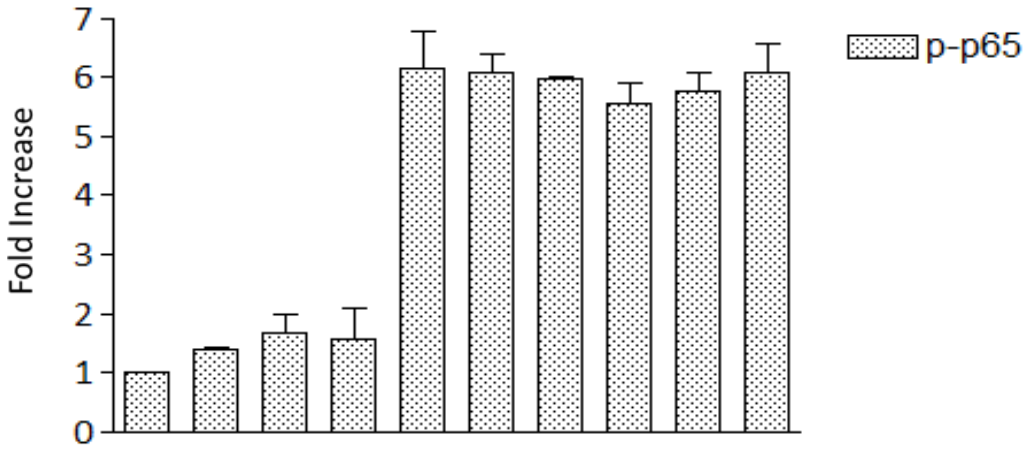
Another marker of activation of canonical NFκB pathway is the phosphorylation of p65. As shown in figure 3.9, TNF-α stimulated p65 phosphorylation was not altered by Adv. DN-IKKα suggesting that SU1007 and SU1010 should not affect this parameter if selective for IKKα. Indeed figure 4.5 demonstrates that whilst TNF-α significantly enhanced p65 phosphorylation, increasing concentrations of SU1007 did not affect the response. Likewise, the response was not altered in the presence of SU1010 up to maximum concentration of 30μM (figure 4.6). In addition, neither SU compounds elicited any response in the absence of TNF-α.

A



---	3	10	30	---	0.3	1	3	10	30	SU 1007 (µM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

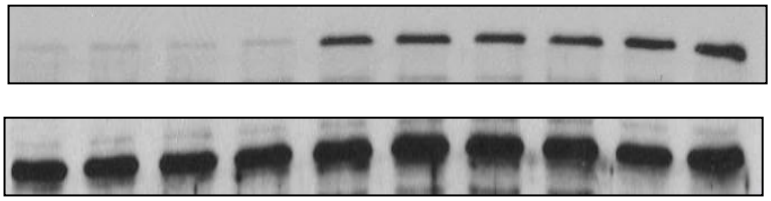
B



---	3	10	30	---	0.3	1	3	10	30	SU 1007 (µM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

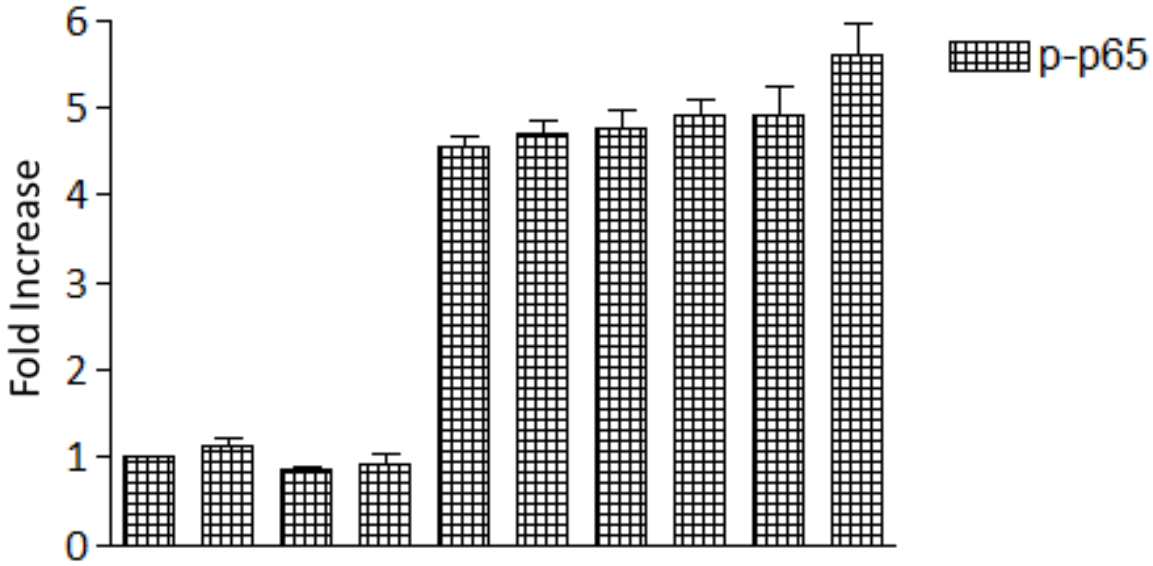
Figure 4.5: The effect of SU1007 upon TNF-α-mediated p65 phosphorylation in HUVECs. Cells were pre-treated with SU1007 for 1h prior to the stimulation with TNF-α (10ng/ml) for 15min. Whole cell lysates were prepared, separated by SDS PAGE and assessed for A) p-p65 (65kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) % expression by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments.

A



---	3	10	30	---	0.3	1	3	10	30	SU 1010 (μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

B



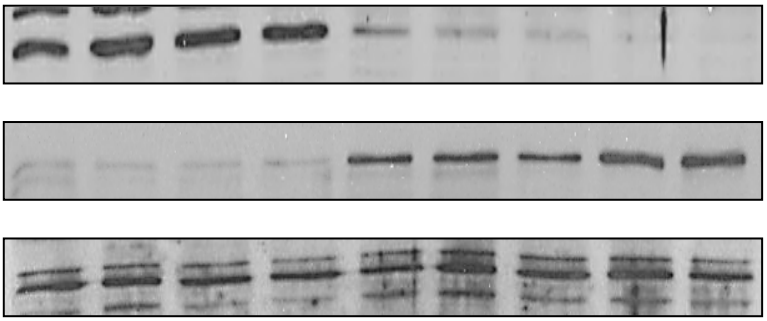
---	3	10	30	---	0.3	1	3	10	30	SU 1010 (μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

Figure 4.6: The effect of SU1010 upon TNF-α-mediated p65 phosphorylation in HUVECs. Cells were pre-treated with SU1010 for 1h prior to the stimulation with TNF-α (10ng/ml) for 15min. Whole cell lysates were prepared, separated by SDS PAGE and assessed for A) p-p65 (65kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) % expression by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments.

4.3.3 Characterisation of the effect of SU compounds on IL-1β induced IκB-α or p65 phosphorylation in HUVECs

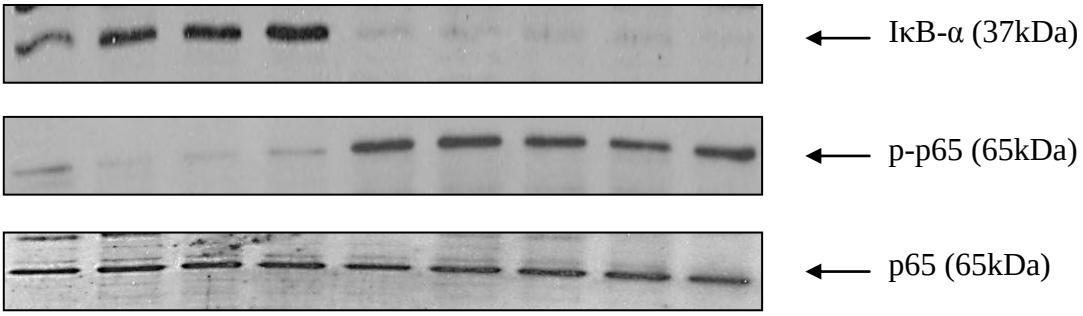
In order to exclude that the lack of effect of SU1007 and SU1010 were due to the specific use of TNF-α, the cytokine IL-1β was examined as a comparator. In figure 4.7, HUVECs were pre-treated with SU1007 or SU1010 for 1h prior to stimulation with IL-1β. Stimulation with 10ng/ml of IL-1β resulted in a marked increase in IκB-α degradation after 30min. Again, a maximum concentration of either SU compound (30μM) did not affect IL-1β induced IκB-α loss and p65 phosphorylation.

A



---	3	10	30	---	1	3	10	30	SU 1007 (μM)
---	---	---	---	+	+	+	+	+	IL-1β (10ng/ml)

B



---	3	10	30	---	1	3	10	30	SU 1010 (μM)
---	---	---	---	+	+	+	+	+	IL-1β (10ng/ml)

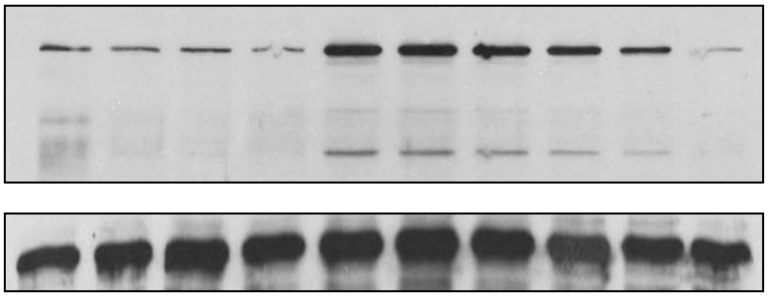
Figure 4.7: The effect of SU1007 and SU1010 upon IL-1β-mediated IκB-α degradation and p65 phosphorylation in HUVECs. Cells were pre-treated with A) SU1007 or B) SU1010 for 1h prior to the stimulation with TNF-α (10ng/ml) for 15min. Whole cell lysates were prepared, separated by SDS PAGE and assessed for IκB-α (37kDa), p-p65 (65kDa) and p65 (65kDa) as outlined in section 2.3. The result is representative of 1 experiment.

4.4 Characterisation of SU compounds on TNF-α induced p100 processing in HUVECs

Figure 4.8 shows the effect of SU1007 on TNF-α induced p100 processing in HUVECs. Alone, SU1007 did not induce any increase in p100 processing. As shown in figures 3.11 and 3.12, TNF-α significantly induced p100 processing resulting in an approximate 2 fold increase in p52 formation (Fold stim = 2.08 ± 0.32, P<0.01). With increasing concentrations of SU1007, p52 formation gradually decreased with significance achieved at 10 and 30μM (SU1007 10μM: Fold increase = 1.04 ± 0.15, P<0.01 and SU1007 30μM: fold increase = 0.51 ± 0.05, P<0.01). This inhibition of p52 was accompanied by a significant reduction in the expression of p100 (TNF-α: Fold stim = 3.32 ± 0.01, SU1007 10μM: Fold stim= 2.24 ± 0.09, p<0.05 and SU1007 30μM: Fold stim = 0.95 ± 0.58, p<0.01). Examination the ratio of p100/p52 gave an inconsistent pattern with no significant change. Interestingly, pre-treatment with the non-selective IKK inhibitor BMS-345541 (30μM) also demonstrated a significant reduction of both p52 formation (Fold stim = 0.28 ± 0.002, P<0.01) and p100 (Fold stim = 0.39 ± 0.06, P<0.01).

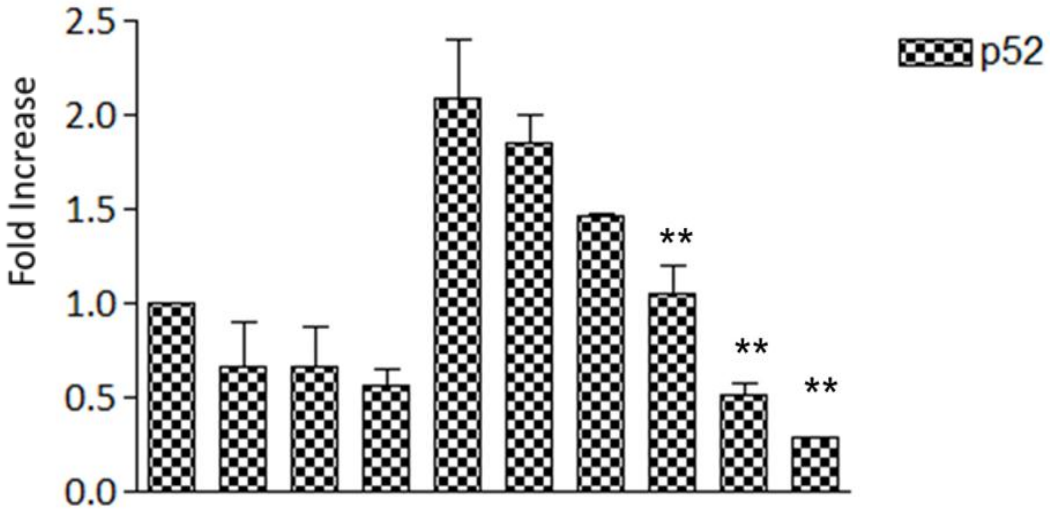
Similar to the effect of SU1007, pre-treatment with SU1010 reduced TNF-α stimulated p52 formation, gradually decreasing the response over the low μM range (figure 4.9). Significant inhibition was again obtained at 10 and 30μM. TNF-α alone significantly induced p100 processing resulting in an approximate 2 fold increase in p52 formation (Fold stim= 2.01 ± 0.11). With increasing concentrations of SU1010, p52 formation gradually and significantly decreased with significance achieved at 10 and 30μM (SU1010 10μM: Fold stim= 0.80 ± 0.34, P<0.05 and 30μM: Fold stim= 0.30 ± 0.25, P<0.01). Similarly, SU1010 also reduced p100 expression (TNF-α: Fold stim= 1.85 ± 0.21, SU1010 30μM: Fold stim= 0.30 ± 0.25, P<0.01). As with the previous experiment, pre-treatment with 10μM of the non-selective IKK inhibitor BMS-345541 also resulted in a significant reduction in both p52 formation (Fold stim= 0.86 ± 0.04, P<0.05) and p100 (Fold stim= 0.67 ± 0.09, p<0.01), relative to TNF-α.

A



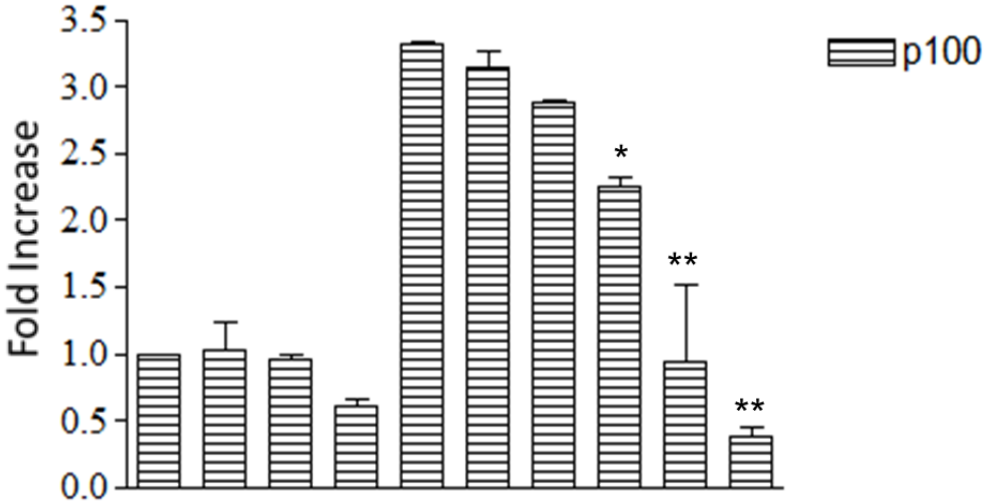
---	3	10	30	---	1	3	10	30	---	SU 1007 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

B



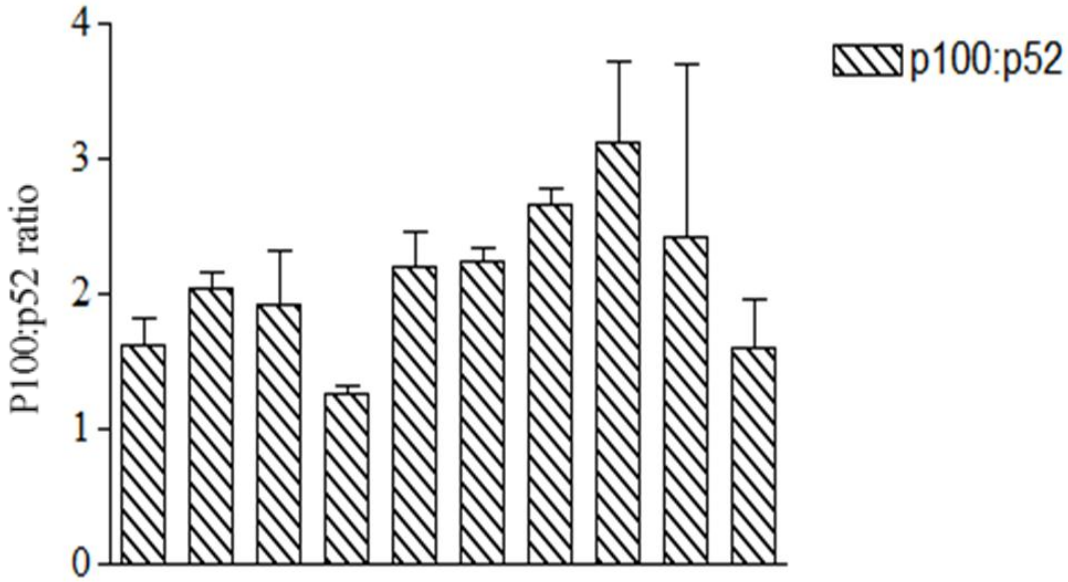
---	3	10	30	---	1	3	10	30	---	SU 1007 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

C



---	3	10	30	---	1	3	10	30	---	SU 1007 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

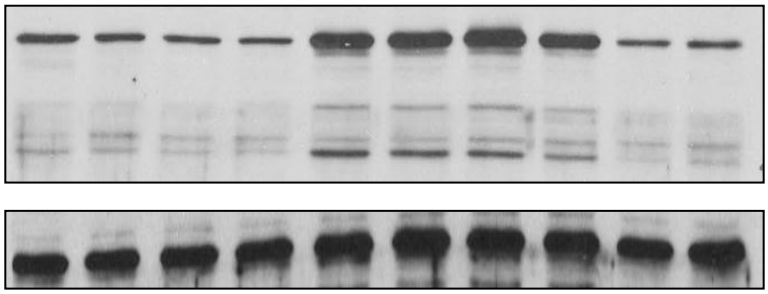
D



---	3	10	30	---	1	3	10	30	---	SU 1007 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

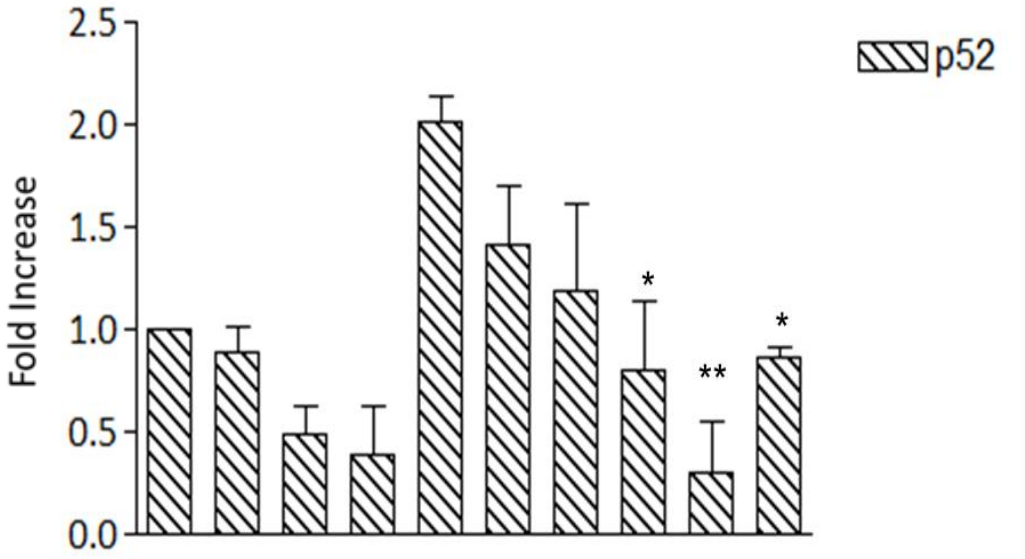
Figure 4.8: The effect of SU1007 upon TNF-α-mediated p100 processing in HUVECs. Cells were pre-treated with SU1007 for 40h prior to stimulation with TNF-α (10ng/ml) for 14h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for for A) p100 (100kDa), p52 (52kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) Fold stimulation of p52 and C) p100 are measured by scanning densitometry and D) The ratio of p100:p52, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments. *P<0.05, **P<0.01 compared with agonist stimulated control.

A



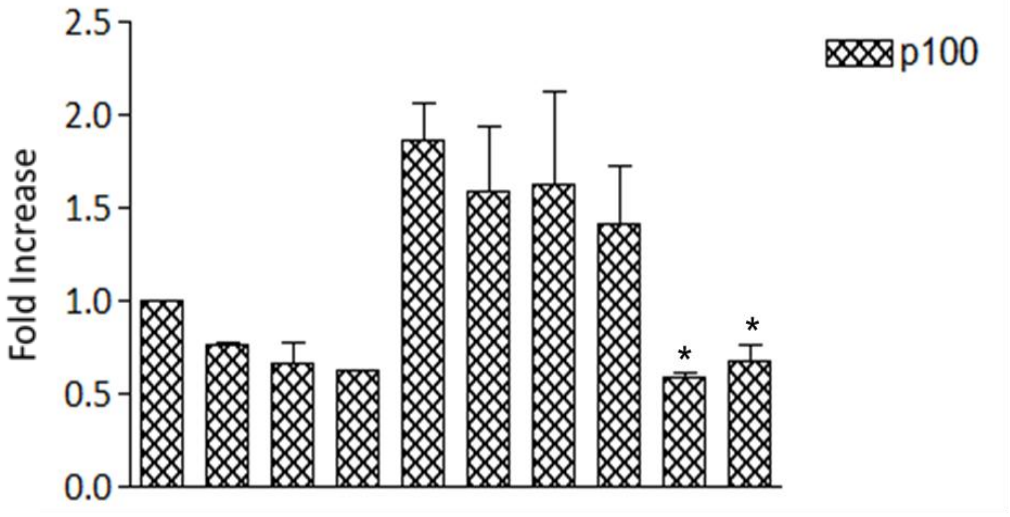
---	3	10	30	---	1	3	10	30	---	SU 1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

B



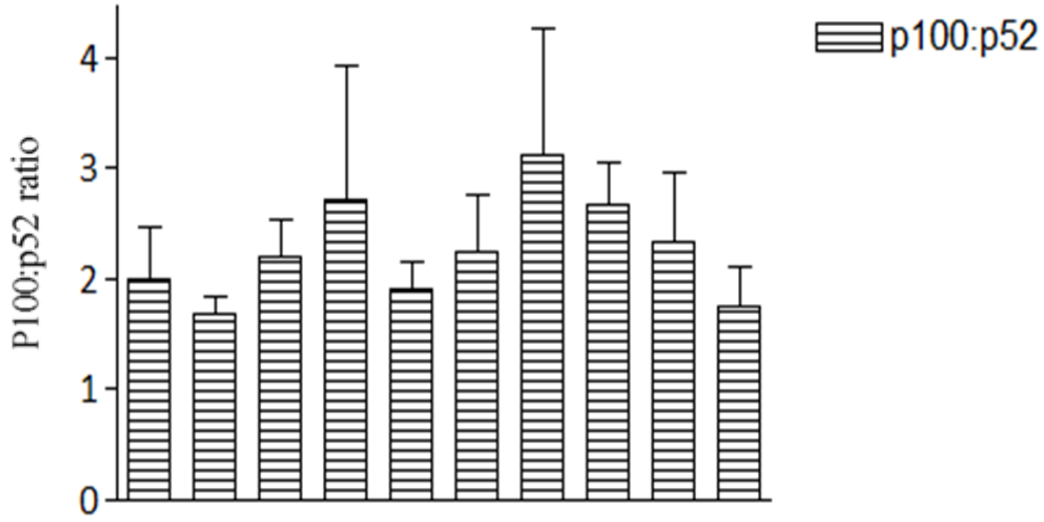
---	3	10	30	---	1	3	10	30	---	SU1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

C



---	3	10	30	---	1	3	10	30	---	SU1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

D

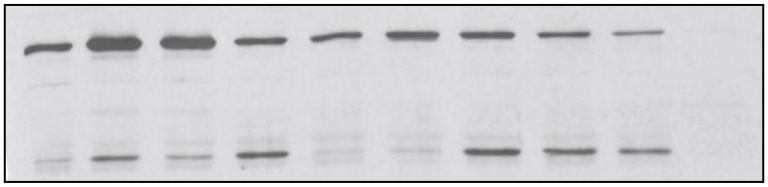


---	3	10	30	---	1	3	10	30	---	SU 1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

Figure 4.9: The effect of SU1010 upon TNF-α-mediated p100 processing in HUVECs. Cells were pre-treated with SU1010 for 40h prior to stimulation with TNF-α (10ng/ml) for 14h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) p100 (100kDa), p52 (52kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) Fold stimulation of p52 and C) p100 are measured by scanning densitometry and D) The ratio of p100:p52, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments. *P<0.05, **P<0.01 compared with agonist stimulated control.

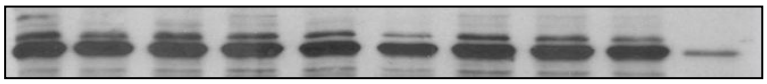
As demonstrated in the previous chapter, Adv. DN-IKKβ enhanced rather than decreased TNF-α induced p100 processing (Figure 3.14 and 3.15). Having established that in figure 4.10 panel A, TNF-α induced p52 formation (3.45 fold) in the presence of Adv. DN-IKKβ caused increased p52 formation (4.87 fold), whilst increasing the concentration of SU1007 with fixed concentration of Adv. DN-IKKβ resulted in decreased in p52 formation (SU1007 10μM: Fold stim = 3.75, 30μM: Fold stim = 0.26). As shown in panel B, TNF-α induced p52 formation (2.49 fold) in the presence of Adv. DN-IKKβ caused increased p52 formation (3.5 fold). Similarly, increasing the concentration of SU1010 also reduced Adv. DN-IKKβ enhanced p52 formation (SU1010 10μM: Fold stim = 0.90, 30μM: Fold stim = 0.32).

A



p100 (100kDa)

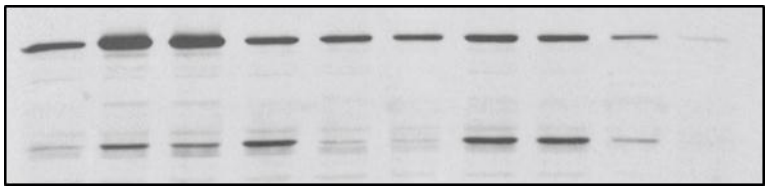
p52 (52kDa)



p65 (65kDa)

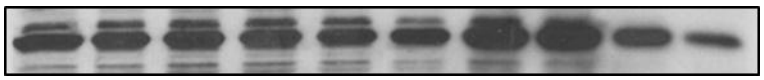
---	---	---	---	30	30	1	3	10	30	SU 1007 (μM)
---	+	+	+	---	+	+	+	+	+	TNF-α (10ng/ml)
--	---	+	---	---	---	---	---	---	---	DN-IKKα(300ofu)
--	---	---	+	---	---	+	+	+	+	DN-IKKβ (300pfu)

B



p100 (100kDa)

p52 (52kDa)



p65 (65kDa)

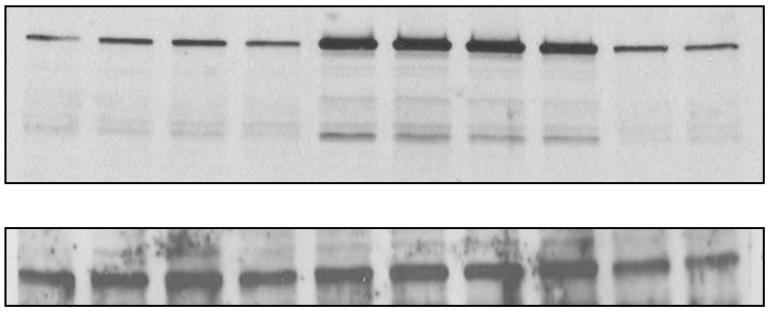
---	---	---	---	30	30	1	3	10	30	SU 1010 (μM)
---	+	+	+	---	+	+	+	+	+	TNF-α (10ng/ml)
--	---	+	---	---	---	---	---	---	---	DN-IKKα(300ofu)
--	---	---	+	---	---	+	+	+	+	DN-IKKβ (300pfu)

Figure 4.10: The effect of SU compounds on Adv. DN-IKKβ pre-treated on p100 processing. Cells were pre-treated with Adv. DN-IKKβ (300pfu) for 40h and/or additionally SU1007 (1-30μM) or SU1010 (1-30μM) for 1h prior to the stimulation of TNF-α for 14h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for p100 (100kDa), p52 (52kDa) and p65 (65kDa) as outlined in section 2.3. The result is representative of 1 independent experiment.

In preliminary experiments, two other IKK selective inhibitors, SU1028 and SU1053, were examined for effects on p100 processing. As shown in figure 4.11, TNF-α induced p100 processing and formation of p52. Concentrations of 3 and 10μM of SU1028 caused an approximate 30% and 36% inhibition of the TNF-α control response, respectively. A dramatic reduction (83.2%) in the response was elicited by a maximum concentration (30μM) of the compound. A similar response was observed for SU1053 with 3 and 10μM of SU1053 causing an approximate 16% and 36% inhibition of the response respectively, whilst the maximum concentration of SU1053 (30μM) caused inhibition of approximately 70%. For both compounds, p100 expression was similarly affected.

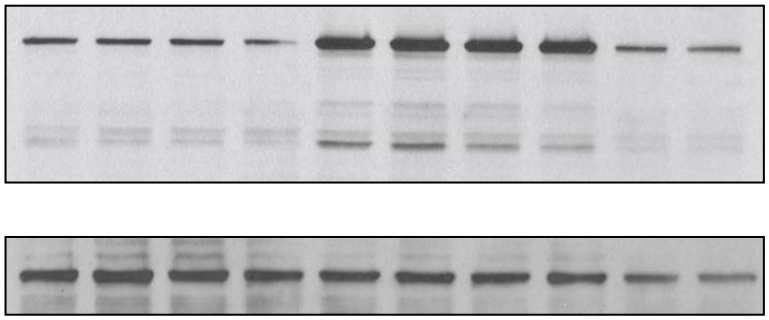
To further confirm that p100 processing was IKKα dependent and that SU compounds had an effect on IKKα, the effect of a selective IKKβ inhibitor (SU937) on p100 processing was investigated. In figure 4.12, TNF-α was shown to induce p100 processing and the effect was not altered in the presence of DMSO. However, increasing concentrations of SU937 showed no effect on TNF-α induced p100 processing. As a comparison, expression of DN-IKKα reduced p52 formation whilst DN-IKKβ slightly enhanced the processing.

A



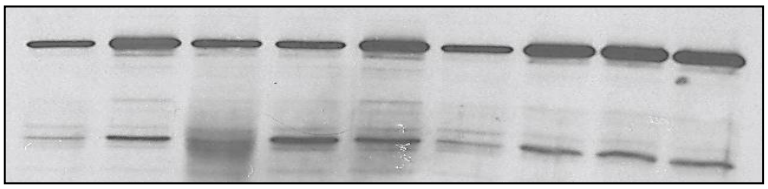
---	3	10	30	---	1	3	10	30	---	SU 1028 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

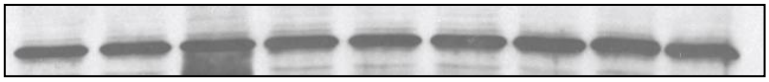
B



---	3	10	30	---	1	3	10	30	---	SU 1053 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (10ng/ml)

Figure 4.11: The effect of SU1028 and SU1053 upon TNF-α-mediated p100 processing in HUVECs. Cells were pre-treated with either SU1028 or SU1053 for 40h prior to stimulation with TNF-α (10ng/ml) for 14h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) p100 (100kDa), p52 (52kDa) and p65 (65kDa) as outlined in section 2.3. The result is representative of 1 independent experiment.



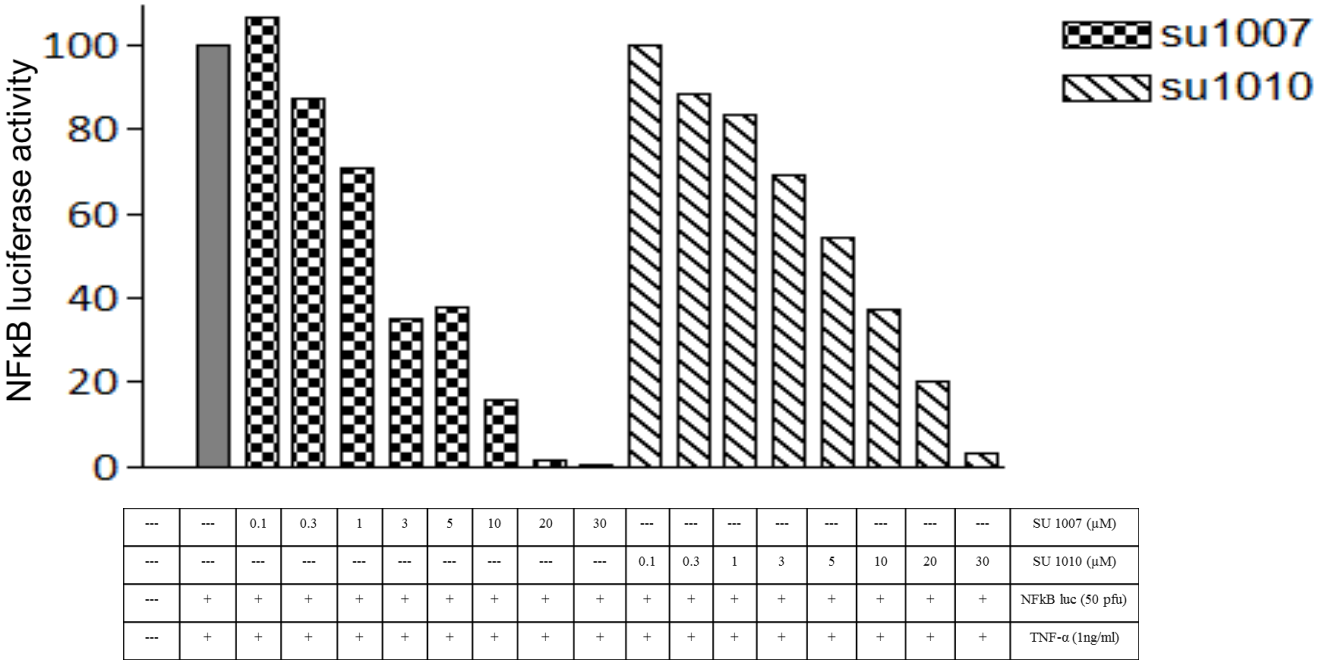


---	---	---	---	---	30	3	10	30	SU 937 (μM)
---	---	---	---	+	---	---	---	---	DMS0 (10μM)
---	+	+	+	+	---	+	+	+	TNF-α (10ng/ml)
--	---	+	---	---	---	---	---	---	DN-IKKα(300pfu)
--	---	---	+	---	---	---	---	---	DN-IKKβ (300pfu)

Figure 4.12: The effect of SU937 upon TNF-α-mediated p100 processing in HUVECs. Cells were pre-treated with SU937 for 40h prior to stimulation with TNF-α (10ng/ml) for 14h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) p100 (100kDa), p52 (52kDa) and p65 (65kDa) as outlined in section 2.3. The result is representative of 1 independent experiment.

4.5 Characterisation of the effect of SU compounds in the regulation of TNF-α induced NFκB reporter gene activity in HUVECs

The previous chapter (figure 3.17) showed that Adv.DN-IKKα partially inhibited either TNF-α or IL-1β stimulated p65 transcription activity in HUVECs. Therefore the SU compounds might be able to also inhibit the transcriptional activity and that was examined as shown in figure 4.13. As predicted, increasing concentrations of SU1007 gradually decreased NFκB reporter activity. Although the effect of SU1007 on NFκB transcription activity shows a concentration dependent manner, 3 and 5μM of SU1007 gave a similar inhibitory effect whilst 10μM caused further inhibition and a maximum concentration (30μM) abolished the response. The IC_{50} value for SU1007 was 8.04μM and the IC_{50} value for SU1010 is 2.31μM.



---	---	0.1	0.3	1	3	5	10	20	30	---	---	---	---	---	---	---	---	SU 1007 (µM)
---	---	---	---	---	---	---	---	---	---	0.1	0.3	1	3	5	10	20	30	SU 1010 (µM)
---	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NFkB luc (50 pfu)
---	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	TNF-α (1ng/ml)

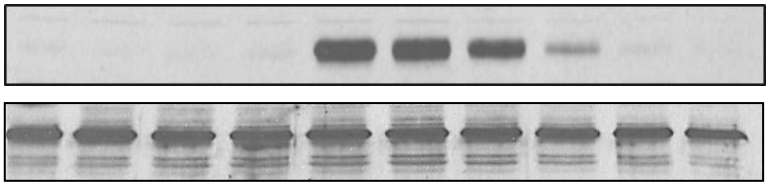
Figure 4.13: The effect of SU1007 and SU1010 upon TNF-α-mediated NFκB luciferase activity in HUVECs. Cells were pre-treated with SU1007 or SU1010 for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Cell lysates were then measured for luciferase activity as previously described in section 2.6. Data shown is expressed as % agonist stimulation. The result is representative of 1 experiment.

4.6 Characterisation of SU1007 and SU1010 on TNF-α induced ICAM-1 and VCAM-1 expression in HUVECs

The previous chapter demonstrated that IKKα plays a role in the regulation of p65-dependent ICAM-1 and VCAM-1 expression. Blocking IKKα by Adv. DN-IKKα or siRNA IKKα leads to a partial reduction of both TNF-α or IL-1β induced ICAM-1 and VCAM-1 in HUVECs (figure 3.21, 3.22 and 3.23). The SU compounds were tested to determine if they could cause a similar inhibitory effect. Figure 4.14 demonstrates the effect of SU1007 on TNF-α induced ICAM-1 expression in HUVECs. TNF-α significantly induced ICAM-1 expression (Fold increase= 58.3 ± 4.72, $P<0.01$). The response was not altered by the lowest concentration of SU1007 (1μM), but thereafter the inhibitory effect was concentration dependent. As little as 3μM of SU1007 reduced the expression of ICAM-1 by about 40% (Fold increase= 34.5 ± 13.5, $P<0.05$), while 10μM reduced the response by approximately 82% (Fold increase= 10.4 ± 5.5, $P<0.01$). A maximum concentration (30μM) of SU1007 virtually abolished ICAM-1 expression. Similar to the combined adenoviral mediated inhibition of both IKKα and β (figure 3.21, 3.22 and 3.23), the non-selective IKK inhibitor BMS-345541 also abolished the ICAM-1 expression (Fold increase= 3.04 ± 0.18, $P<0.01$).

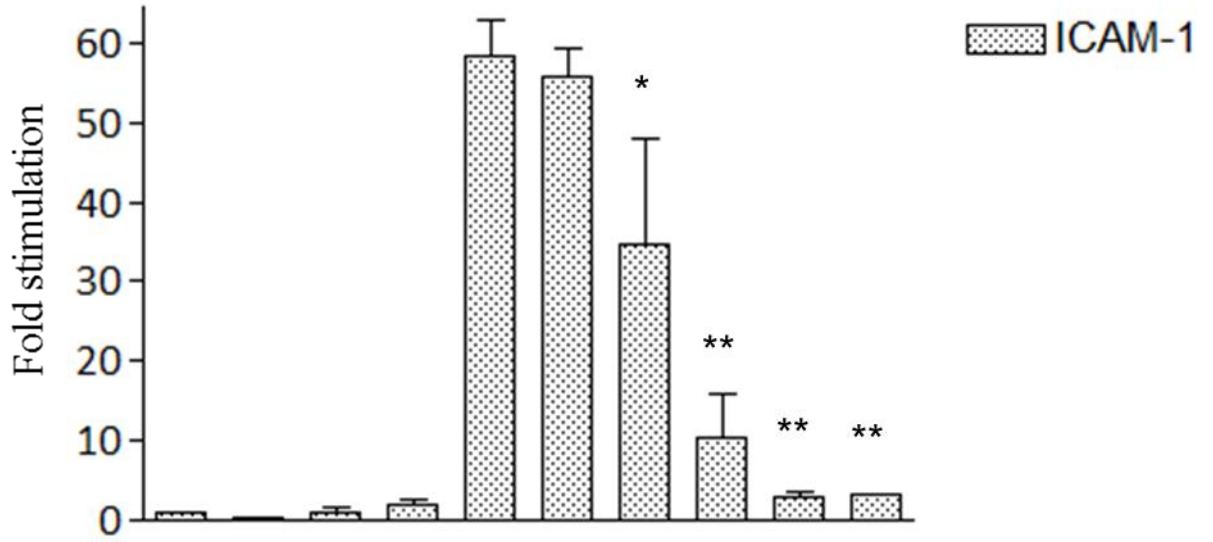
Likewise, SU1010 also caused a concentration-dependent inhibition of TNF-α stimulated ICAM-1 expression in HUVECs (Figure 4.15). Again, the presence of the lowest concentration (1μM) of the compound did not affect the response, however, 3μM gave around a 39% reduction (Fold increase= 31.9 ± 0.52, $P<0.05$) and over 80% at 10μM (Fold increase= 6.96 ± 0.34, $P<0.01$). A maximum concentration (30μM) of SU1010 virtually abolished ICAM-1 expression.

A



---	3	10	30	---	1	3	10	30	---	SU 1007 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

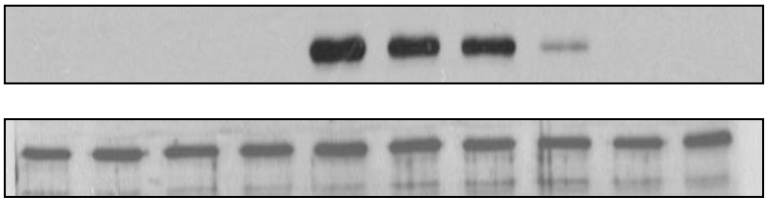
B



---	3	10	30	---	1	3	10	30	---	SU 1007 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

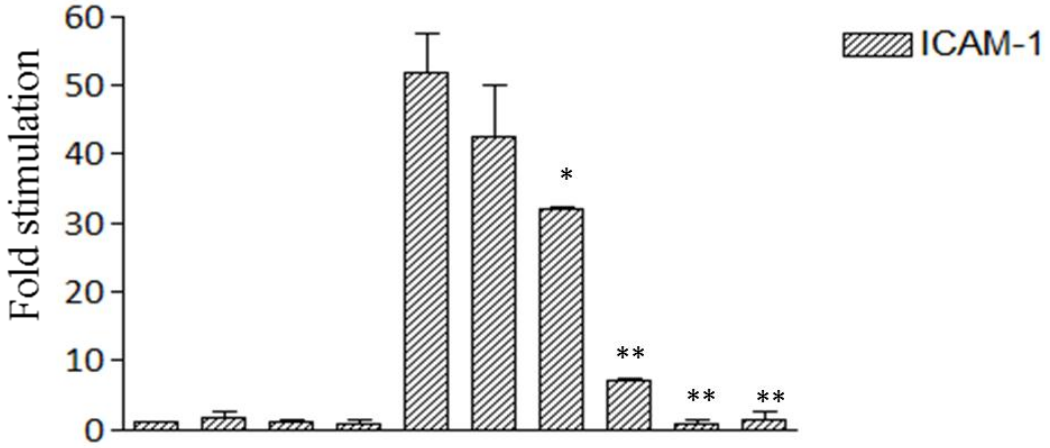
Figure 4.14: The effect of SU1007 upon TNF-α-mediated ICAM-1 expression in HUVECs. Cells were pre-treated with SU1007 for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) ICAM-1 (90kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) Fold stimulation of ICAM-1 expression by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments. *P<0.05, **P<0.01 compared with agonist control.

A



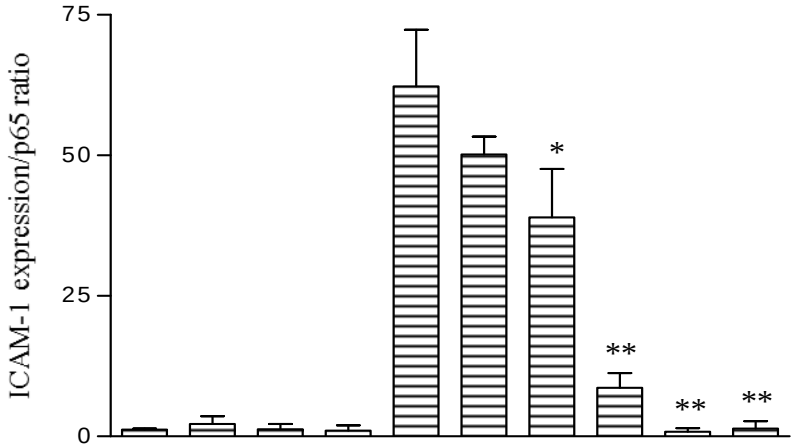
---	3	10	30	---	1	3	10	30	---	SU 1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

B



---	3	10	30	---	1	3	10	30	---	SU 1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

C



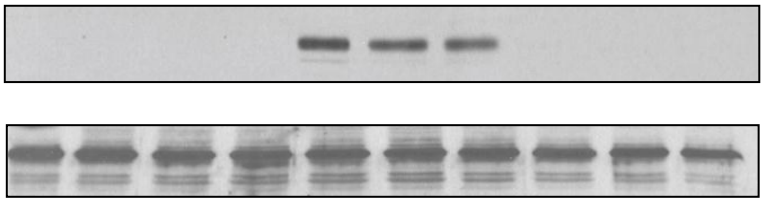
---	3	10	30	---	1	3	10	30	---	SU 1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

Figure 4.15: The effect of SU1010 upon TNF-α-mediated ICAM-1 expression in HUVECs. Cells were pre-treated with SU1010 for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) ICAM-1 (90kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) Fold stimulation of ICAM-1 expression and C) ICAM-1/p65 ratio by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments. *P<0.05, **P<0.01 compared with agonist control.

Similar to ICAM-1, both SU compounds showed inhibitory effects on TNF-α induced VCAM-1 expression. As shown in figure 4.16, TNF-α significantly stimulated VCAM-1 expression over 8h (Fold increase= 28.6 ± 4.60, P<0.01). As little as 3μM of SU1007 reduced the expression of VCAM-1 by approximately 40% (Fold increase = 16.5 ± 3.80, P<0.05), while 10μM of SU1007 elicited up to 90% of the inhibitory effect (Fold increase = 2.89 ± 0.47, P<0.01) and 30μM of SU1007 completely abolished the expression of VCAM-1 (Fold increase = 1.31 ± 0.43, P<0.01). As expected, inhibition of both IKKα and IKKβ by BMS-345541 resulted in virtual abolition of the response (Fold increase = 1.52 ± 0.14, P<0.01).

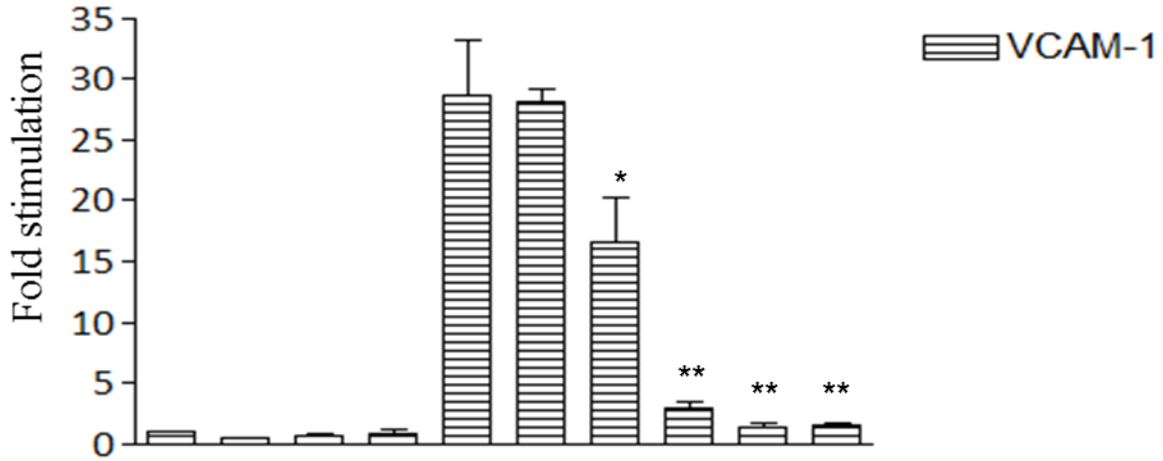
The effect of SU1010 on VCAM-1 expression is illustrated in figure 4.17. TNF-α (1ng/ml) induced VCAM-1 expression significantly (Fold increase = 16.0 ± 4.1, P<0.01). Pre-treatment with SU1010 caused a concentration dependent reduction in VCAM-1 expression which was significant with a concentration as low as 3μM (Fold increase= 9.42 ± 0.57, P<0.05), and gave as much as 90% inhibition with 10μM (Fold increase= 1.42 ± 0.54, P<0.01) of SU1010. A maximum concentration of SU1010 (30μM) completely abolished the expression of VCAM-1 (Fold increase= 0.87 ± 0.08, P<0.01). As expected, inhibition of both IKKα and IKKβ by BMS-345541 (30μM) resulted in almost abolition of the response (Fold increase= 1.52 ± 0.14, P<0.01).

A



---	3	10	30	---	1	3	10	30	---	SU 1007 (µM)
---	---	---	---	---	---	---	---	---	+	BMS (10µM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

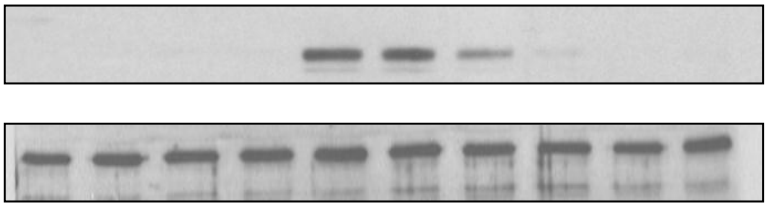
B



---	3	10	30	---	1	3	10	30	---	SU 1007 (µM)
---	---	---	---	---	---	---	---	---	+	BMS (10µM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

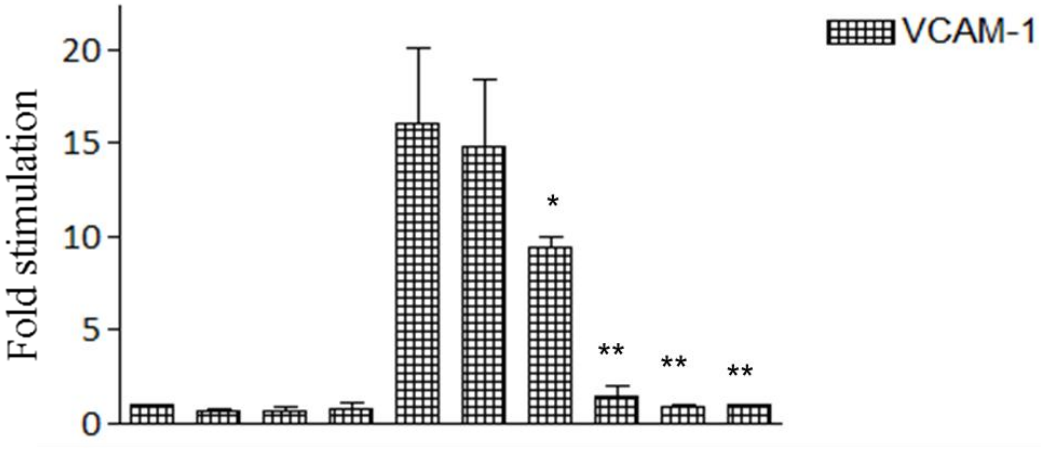
Figure 4.16: The effect of SU1007 upon TNF-α-mediated VCAM-1 expression in HUVECs. Cells were pre-treated with SU1007 for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) VCAM-1 (110kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) Fold stimulation of VCAM-1 expression by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments. *P<0.05, **P<0.01 compared with agonist control.

A



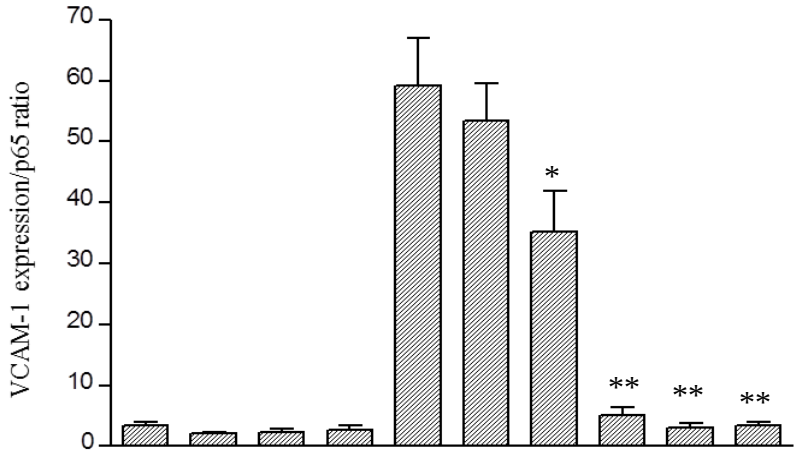
---	3	10	30	---	1	3	10	30	---	SU 1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

B



---	3	10	30	---	1	3	10	30	---	SU 1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

C



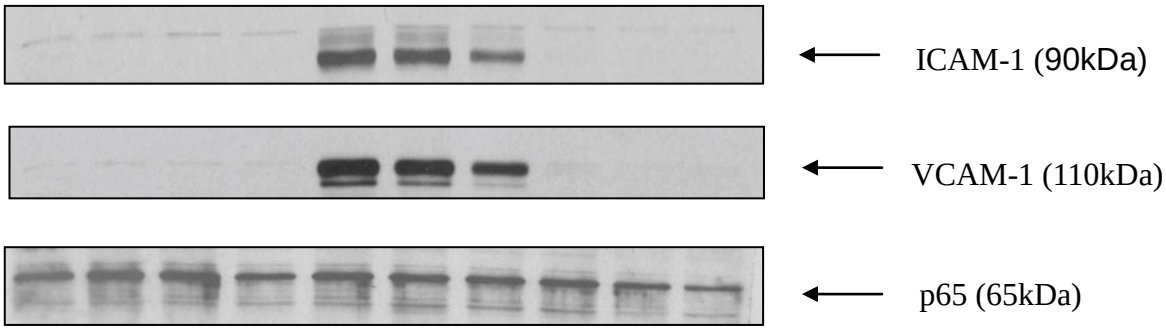
---	3	10	30	---	1	3	10	30	---	SU 1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

Figure 4.17: The effect of SU1010 upon TNF-α-mediated VCAM-1 expression in HUVECs. Cells were pre-treated with SU1010 for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for A) VCAM-1 (110kDa) and p65 (65kDa) as outlined in section 2.3. Blots were quantified for B) Fold stimulation of VCAM-1 expression and C) VCAM-1/p65 ratio by scanning densitometry, each value represents the mean ± S.E.M. The results are representative of 3 independent experiments. *P<0.05, **P<0.01 compared with agonist control.

In preliminary experiments, the effect of SU1028 and SU1053 on adhesion molecule expression was examined in HUVECs (figure 4.18). TNF-α induced a significant increase in ICAM-1 over a 8h period with a fold stim approximate 23 to 26. Both SU1028 and SU1053 also caused a significant inhibition as demonstrated in figure 4.18. However whilst 3μM of SU1028 caused an approximate 50% reduction (Fold stim = 14.1) of the response and 10μM was sufficient to abolish ICAM-1 expression. SU1053 showed a weaker inhibitory effect on the response with 3μM causing only 26% reduction in ICAM-1 expression (Fold stim = 17.4), whilst 10μM elicited a 70% reduction of the response (Fold stim = 7.29). Like other SU compounds, a concentration of 30μM effectively abolished the expression of ICAM-1.

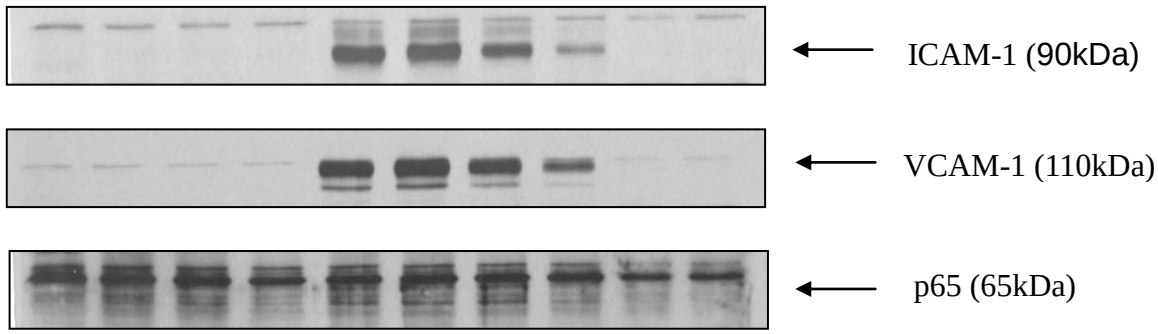
Likewise, both SU1028 and SU1053 also inhibited the TNF-α induced VCAM-1 expression. VCAM-1 expression markedly increased following TNF-α stimulation (Fold increase = 20-33). SU1028 (3 μM) caused 37% drop in the response (Fold increase = 21.1) and 10μM almost abolished the expression. Again, SU1053 also showed a weaker inhibitory effect on VCAM-1 expression. SU1053 (3μM) caused around 15% reduction (Fold increase = 17.3) and10μM elicited only 50% drop of the response (Fold increase = 10.23), whilst 30μM again completely inhibited the VCAM-1 expression.

A



---	3	10	30	---	1	3	10	30	---	SU 1028 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

B



---	3	10	30	---	1	3	10	30	---	SU 1053 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	TNF-α (1ng/ml)

Figure 4.18: The effect of SU1007 and SU1010 upon TNF-α-mediated ICAM-1 and VCAM-1 expression in HUVECs. Cells were pre-treated with A) SU1028 or B) SU1053 for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for ICAM-1 (90kDa), VCAM-1 (110kDa) and p65 (65kDa) as outlined in section 2.3. The result is representative of 1 experiment.

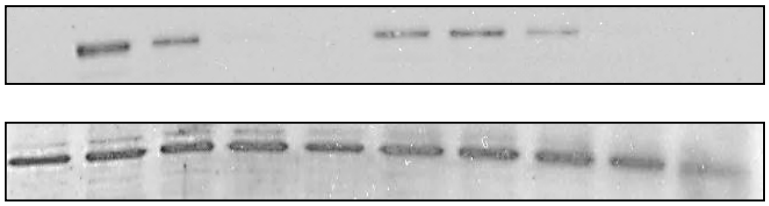
4.7 Characterisation of SU compounds in combination with Adv. DN-IKKβ on TNF-α induced ICAM-1 expression on HUVECs

The previous chapter demonstrated that a combination of Adv. DN-IKKα and β induced a greater inhibitory effect on either ICAM-1 and VCAM-1 expression than inhibition of IKKα or β alone (figure 3.21, 3.22). As the SU compounds have been shown to inhibit both ICAM-1 and VCAM-1 expression which may be mediated by an IKKα dependent effect, they may also be able to enhance the inhibitory effect of Adv. DN-IKKβ**.** As figure 4.19 shows pre-treatment with SU1007 inhibited TNF-α induced ICAM-1 expression in the expected concentration dependent manner. Whilst 1μM of SU1007 was ineffective, as little as 3μM SU1007 caused around 50% reduction in the response. Adv. DN-IKKβ (75pfu) alone induced around 40% reduction of the response, whilst a combination of 1μM SU1007 with Adv. DN-IKKβ however did not elicit any stronger inhibitory effect than Adv. DN-IKKβ alone. In panel B, SU1010 was examined. Adv. DN-IKKβ (75pfu) alone induced an approximate 60% reduction in TNF-α induced ICAM-1, whilst again 1μM of SU1010 did not enhance the inhibitory effect of Adv. DN-IKKβ. These results together suggest that dual treatments with SU compounds with Adv. DN-IKKβ did not cause synergistic inhibitory effect, rather than a combination of 3μM of the SU compounds with Adv. DN-IKKβ seemed to cause an additive inhibitory effect.

4.8 Characterisation of SU compounds on IL-1β induced ICAM-1 and VCAM-1 in HUVECs

As demonstrated in the previous chapter, expression of Adv. DN-IKKα caused a partial inhibition of IL-1β stimulated ICAM-1 and VCAM-1 expression. Therefore it is possible that a similar inhibitory effect might be observed following pre-treatment with SU compounds. As expected, figure 4.20 panel A shows that SU1007 inhibited both IL-1β induced ICAM-1 and VCAM-1 expression in concentration dependent manner. As with TNF-α, reduction was observed over the low micromolar range (1 to 30 μM) with significant and considerable inhibition of both adhesion molecules at 10 and 30μM. A similar result was obtained following pre-treatment with SU1010 (Panel B), although overall this compound gave slightly stronger inhibition of either ICAM-1 or VCAM-1.

A

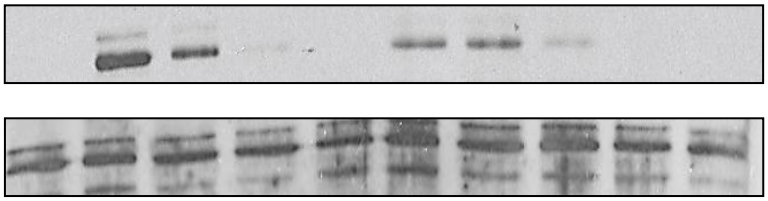


ICAM-1 (90kDa)

p65 (65kDa)

---	---	3	10	30	---	1	3	10	30	SU 1007 (μM)
---	---	---	---	---	+	+	+	+	+	DN-IKKβ (75pfu)
---	+	+	+	+	+	+	+	+	+	TNF-α (1ng/ml)

B



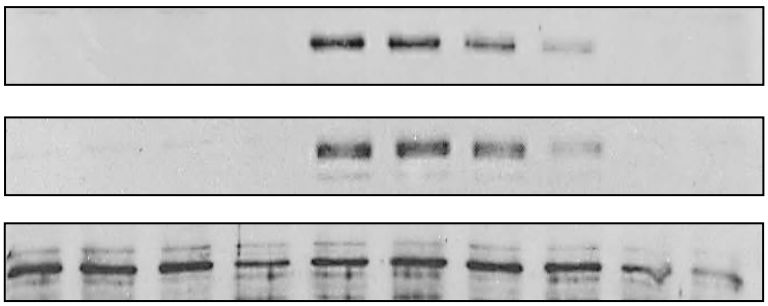
ICAM-1 (90kDa)

p65 (65kDa)

---	---	3	10	30	---	1	3	10	30	SU 1010 (μM)
---	---	---	---	---	+	+	+	+	+	DN-IKKβ (75pfu)
---	+	+	+	+	+	+	+	+	+	TNF-α (1ng/ml)

Figure 4.19: The compound effects of SU1007 and SU1010 with Adv. DN-IKKβ overexpression upon TNF-α-mediated ICAM-1 expression in HUVECs. Cells were pre-treated with A) SU1007 or B) SU1010 and Adv. DN-IKKβ for 40h prior to stimulation with TNF-α (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for ICAM-1 (90kDa) and p65 (65kDa) as outlined in section 2.3. The result is representative of 1 experiment.

A



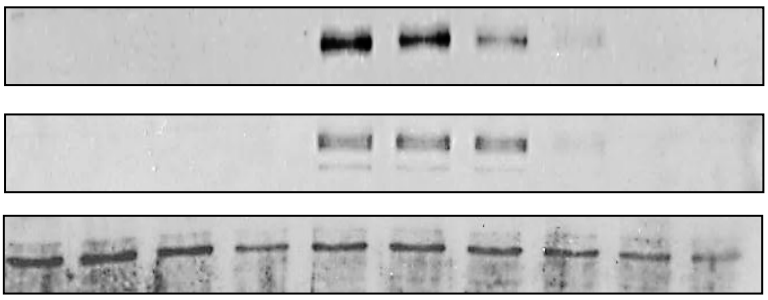
ICAM-1 (90kDa)

VCAM-1 (110kDa)

p65 (65kDa)

---	3	10	30	---	1	3	10	30	---	SU 1007 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	IL-1β (1ng/ml)

B



ICAM-1 (90kDa)

VCAM-1 (110kDa)

p65 (65kDa)

---	3	10	30	---	1	3	10	30	---	SU 1010 (μM)
---	---	---	---	---	---	---	---	---	+	BMS (10μM)
---	---	---	---	+	+	+	+	+	+	IL-1β (1ng/ml)

Figure 4.20: The effect of SU1007 and SU1010 upon IL-1β-mediated ICAM-1 and VCAM-1 expression in HUVECs. Cells were pre-treated with A) SU1007 or B) SU1010 1h prior to stimulation with IL-1β (1ng/ml) for 8h. Whole cell lysates were prepared, separated by SDS-PAGE and assessed for ICAM-1 (90kDa) and VCAM-1 (110kDa) and p65 (65kDa) as outlined in section 2.3. The result is representative of 1 experiment.

4.9 Discussion

As discussed in chapter 1, NFκB is strongly associated with the development of inflammatory diseases including atherosclerosis, rheumatoid arthritis and also cancers. In addition, de Martin and co-workers found that NFκB regulates a series of anti-apoptotic proteins in various cell types including endothelial cells (de Martin et al., 1999, Erl et al., 1999, Stehlik et al., 1998a). Inhibition of NFκB has become a tempting strategy for the therapeutic treatment of these and other diseases. Indeed, the proteosome inhibitor bortezomib has been utilised for the chemotherapeutic treatment of leukaemia and myeloma (Picot et al., 2011, Zheng et al., 2011). A most well-known strategy to inhibit NFκB activity is the use of a proteasome inhibitor to inhibit IκB-α degradation such as MG-132 (Shirley et al., 2005, Snyder et al., 2002) and PS-341 (Gatto et al., 2003, Sunwoo et al., 2001). In addition, the novel NFκB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), is thought to act by inhibiting nuclear translocation of NFκB p65 or DNA binding of NFκB downstream of phosphorylation and degradation of IκB (Ariga et al., 2002, Tatetsu et al., 2005) as a results promotes the induction of apoptosis in chronic lymphocytic leukemia (Horie et al., 2006)

Due to the success of these approaches and the identification of the IKK complex, the development of IKK inhibitors has become the next logical therapeutic target and more specific than those outlined above. The strategy of kinase inhibition has become more commonplace and it is facilitated by new technologies which is allowed rapid advances in compound screening. A considerable number of IKKβ inhibitors have been developed by this process including PS-1145 (Castro et al., 2003) and ML120B (Catley et al., 2006). By comparison with IKKβ, there are only few compounds present which have a selective inhibitory effect on IKKα such as noraristeromycin which was shown to strongly inhibit the kinase activity of IKKα but not IKKβ in HEK293 (Asamitsu et al., 2008) and a peptidyl – glucosamine derivative which inhibited IKKα nuclear translocation (Scotto d'Abusco et al., 2010). Today, there are no commercial IKKα inhibitors in the market.

A series of novel IKKα inhibitors have been developed by the Medicinal Chemistry group in the University of Strathclyde exemplified by prototypic compounds SU1007 and SU1010. These compounds contain an azole ring which specifically binds to the ATP binding domain of the kinase. This is a common mode of action for many kinase inhibitors (i.e.small

molecule inhibitors). Initially, an *in vitro* kinase assay successfully demonstrated both SU1007 and SU1010 have higher affinity for purified IKKα protein rather for IKKβ.

The initial objective was to characterise whether the compounds remain active in a cellular environment. As IKKα plays no role in the canonical NFκB cascade (shown in previous chapter), selective IKKα inhibitors should not influence in TNF-α induced IκB-α loss and p65 phosphorylation. As expected, neither SU1007 nor SU1010 affected these responses, strongly supporting the notion that the compounds do not inhibit IKKβ. However clearly there are a number of issues that need to be addressed in relation to cellular access and drug metabolism which were assessed indirectly. To ensure the compound had sufficient time to access the kinases inside the cell, the pre-treatment period of both compounds was prolonged to 8h. Again, neither SU1007 nor SU1010 had any effect on TNF-α induced IκB-α loss and p65 phosphorylation. In addition, to prevent a strong agonist response masking any inhibitory effect of the compounds, the TNF-α concentration was reduced 10 fold to 1ng/ml for cellular stimulation and again, the response was not altered in the presence of SU1007 and SU1010. Whilst taken together, these data suggest the potential of IKKα selectivity, other approaches could have been considered including *in vitro* kinase assay following immunoprecipitation. However, this procedure is unable to separate the components of the IKK complex and any activity would have been representative of both kinases. In addition, NFκB/DNA binding may also have been used to confirm selectivity. Studies looking at other putative IKKα inhibitors have not demonstrated pathway selectivity (Asamitsu et al., 2008; Scotto d'Abusco et al., 2010) and this may be crucial in eventual clinical use.

As mentioned previously, IKKα plays a role in the regulation of p65 NFκB transcription activity in many cell types, the data in chapter 3 showed that Adv. DN-IKKα partially inhibited either TNF-α or IL-1β induced p65 NFκB transcription activity in HUVECs. As expected, both SU1007 and SU1010 caused a reduction of TNF-α induced p65 NFκB transcription activity with high concentrations (10-30μM) resulting in complete inhibition of the response. These finding are in contrast with the effect of DN-IKKα which had only a partial effect and may suggest that the compounds have an additional action on IKKβ. This is exemplified by SU1007 which gave a biphasic effect. This may reflect the 100 fold selectivity of the compound for IKKα over β. The previous chapter demonstrated the potential of synergy between DN-IKKα and β in causing inhibition of NFκB transcriptional activity. Therefore a lower concentration of the SU compounds which acts to inhibit IKKα

selectively might produce a similar additive effect. In addition, preliminary data (not shown) did reveal that whilst SU1007 and SU1010 did not alter TNF-α induced p65 nuclear translocation, both compounds partially inhibited p65/DNA binding as determined by EMSA (SU1007 3μM: % inhibition = 81%; SU1007 10μM: % inhibition = 64%, SU1010 10μM: % inhibition= 6%; BMS 10μM = 64%). This data supports the possibility of IKKβ dependent actions of the compounds.

As discussed in chapter 1, the non-canonical NFκB pathway is IKKα dependent and the previous chapter also showed that Adv. DN-IKKα or siRNA IKKα inhibited p100 processing, whilst Adv. DN-IKKβ did the opposite. In this chapter, all the selective IKKα inhibitors SU1007, SU1010 and also in preliminary experiments, SU1028 and SU1053 demonstrated concentration-dependent inhibitory effects on TNF-α induced p100 processing. In contrast, the selective IKKβ inhibitor SU937 did not alter the response. These data suggest that the compounds have some selectivity for IKKα. Recent studies identifying two selective IKKα inhibitors did not test whether processing was inhibited, however geldanamycin (GA) also showed inhibitory effect on BAFF induced p100 processing (Qing et al., 2007). However, there are caveats to these conclusions. As well as reducing p52 formation, the compounds had an inhibitory effect on p100 expression, a feature not observed for GA, therefore high concentrations of SU compounds may have small off-target effects which may mask the effects on p52 formation. Nevertheless, the inhibitory effect of SU1007 or SU1010 overwhelmed the enhancement of p100 processing by Adv. DN-IKKβ indicating the SU compounds are effective in the inhibition of the non-canonical NFκB pathway. Interestingly, inhibition of IKKβ by Adv. DN-IKKβ enhanced p100 processing, but SU937 did not give a similar response. In conclusion, although SU compounds may block IKKα, the inhibitory mechanisms involved for Adv. DN-IKKα and SU compounds may not be completely the same.

The SU compounds were also tested on downstream markers ICAM-1 and VCAM-1 which were shown in chapter 3 to be regulated by both IKKα and β. Both compounds at moderate concentrations caused complete inhibition of ICAM-1 and VCAM-1 expression in HUVECs in response to either TNF-α or IL-1β. However these markers were more sensitive to inhibition by the compounds suggesting the potential of IKKα selectivity. It is also possible that inhibition at high concentrations may reflect inhibition of IKKβ which could not be detected by assessing both IκB-α loss and p65 phosphorylation. Similar to Adv. DN-IKKα,

the combination of Adv. DN-IKKβ with SU1007 or SU1010 also generated an additive inhibitory effect on TNF-α induced ICAM-1 expression. In addition, other modified SU compounds including SU1028 and SU1053 also caused inhibition on TNF-α induced ICAM-1 and VCAM-1 expression. Interestingly, SU1053 showed a weaker inhibitory effect than other SU compounds, thus implying SU1053 may be less selective for IKKβ.

The results using SU1007 and SU1010 demonstrated these inhibitory concentrations compared well when examining the effects of other IKK inhibitors on inflammatory protein production. There are many studies showing that NFκB or IKK inhibitors suppress agonist induced ICAM-1 and VCAM-1 expression. For instance, a study showed that cyclopentenone prostaglandins inhibited IKK and NFκB activity and hence the ICAM-1 and VCAM-1 expression in endothelial cells (Zernecke et al., 2003). In addition, the non-selective IKK inhibitor BMS-345541 also inhibited TNF-α induced ICAM-1 and VCAM-1 expression in endothelial cells (MacMaster et al., 2003) which confirms the results in this chapter. Similar results can be observed in other cell types, an IKK inhibitor,2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl-nicotinonitrile (ACHP) also showed inhibitory effects on gene expression of these two adhesion molecules in T-cell leukaemia cells (Sanda et al., 2006). Interestingly, an early study also demonstrated that non-selective IKK inhibitors (BAY11-7082 and BAY11-7085) abrogated TNF-α induced ICAM-1 and VCAM-1 expression in HUVECs (Pierce et al., 1997). The BAY-compounds (5μM) reduced approximately 50% of the adhesion molecule expression. The study also showed that BAY11-7082 and BAY11-7085 reduced swelling in a dose-dependent manner in both the rat carrageenan paw edema assay and in a rat adjuvant arthritis model (Pierce et al., 1997). However, BAY11-7082 may not be the ideal treatment for inflammatory diseases as studies have shown that BAY11-7082 induces apoptosis (Garcia et al., 2005, Mori et al., 2002). Since there is no evidence suggesting that inhibition of IKKα results in apoptosis, it is possible that the SU compounds could have desirable therapeutic effects without causing cell death. This aspect was not examined in the thesis due to time constraints.

Despite the findings in this chapter, it remains difficult to conclusively describe the SU compounds as bonafide selective IKKα inhibitors and if time had allowed a number of other approaches would have been undertaken. This includes analysis of the effect of the compounds on the translocation of IKKα to the nucleus shown to be disrupted by the peptidyl –glucosamine derivative (Scotto d'Abusco et al., 2010). In addition, CHIP could have been

employed to determine if the SU compounds could inhibit IKKα binding to IKKα promoters to reduce gene transcription. Furthermore, other downstream markers of IKKα activity include cellular events associated with angiogenesis (discussed in chapter 1). In preliminary experiments, SU1007 and SU1010 were found to be able to inhibit the migration of HUVECs in a scratch wound healing assay model. Whilst other inhibitory actions of the SU compounds could be involved, there are increasing number of studies which suggest that IKKα plays a role in the regulation of angiogeneic or metastatic gene expression such as IL-8 and MMPs and VEGF (Liekens et al. 2010; Agarwal et al., 2005). Therefore SU compounds may inhibit these angiogenic mediators and hence cell migration through inhibition of IKKα. The SU compounds may be useful tools to reveal the role of IKKα in angiogenesis and may be potentially useful anti-cancer agents.

In conclusion, the SU compounds are stable at 37ºC and they were active inside the HUVECs up to 14h. Analysis of a number of intermediates, suggest they do not conclusively have an inhibitory action on the non-canonical NFκB pathway mediated via IKKα. The SU compounds are nevertheless effective inhibitors of inflammatory mediator expression, therefore they may have potential to reduce inflammations in variety of diseases. In the future, SU compounds could be used in mice models of arthritis to determine if the compounds can reduce swelling of the joint and immunological markers such TH1 cell activation and release of cytokines. A future plan may also focus on cellular invasion to investigate whether MMPs are inhibited by SU compounds. Whether or not these compounds could be used in the context of cardiovascular disease remains uncertain.

CHAPTER 5

GENERAL DISCUSSION

5.1 General Discussion

Based on statistical data from the World Health Organization, an estimated 17.3 million people died from cardiovascular disease (CVD) in 2008 including; heart disease, stroke and atherosclerosis, representing 30% of global deaths. Unfortunately, cardiovascular disease is still the number one killer in the world today. Vascular conditions including multifocal atherosclerosis are key risk factors in the development of heart disease (Barbarash et al., 2011). Atherosclerosis is an inflammatory disorder in which a plaque is formed within the artery wall due to the accumulation of low-density lipoprotein (LDL) within macrophages, the subsequent formation of foam cells and migration of smooth muscle cells into the sub-endothelial layer (discussed in chapter 1). Foam cells themselves can further release pro-inflammatory cytokines to activate the NFκB pathway to increase the expression of adhesion molecule expression to facilitate the migration of macrophages. Oxidised LDL can also release chemokines (i.e. MCP-1) and growth factors such as PDGF resulting in proliferation of smooth muscle cells and transmigration into the sub-endothelial layer at the later stage (Libby et al., 2006). Thus, inhibition of the formation of oxidised LDL has become an important therapeutic target for the treatment of atherosclerosis disease. Statins are effective inhibitors of LDL accumulation and work by blocking the HMG CoA reductase enzyme which in turn limits the synthesis of cholesterol by 35-40% (Capurso, 2001, Libby, 2001). Statins are one of the most successful medicines in the treatment of both primary and secondary cardiac episodes and well tolerated. Nevertheless, statins can be pleiotropic. Although statins reduce smooth muscle proliferation which can slow down the progression of atherosclerosis (Bellosta et al., 2004), such an action also affects the synthesis of interstitial collagen which is responsible for the integrity of the plaque's fibrous cap and may leads to rupture and fatal thrombosis (Davies et al., 1993, van der Wal et al., 1994). Therefore despite the obvious benefit of statins and similar drugs, there is still a need for new and improved therapies.

Since atherosclerosis is essentially an inflammatory disease, one pathway which may be a good target for the development of such drugs is Nuclear Factor Kappa B. Studies have shown that non-selective NFκB inhibitors such as PDTC can effectively reduce plaque sizes in the aortic lumen of mice (Jawien et al., 2005). Indeed, inhibition of the canonical NFκB pathway is recognised to have a number of outcomes which would make new drug design attractive. This includes a decrease in pro-inflammatory cytokine production including TNF-

α, IL-1, IL-6 and IL-8, a reduction of inflammatory mediator expression, for example COX-2, adhesion molecules ICAM-1 and VCAM-1 or chemoattractant (i.e. MCP-1) and finally reversal of smooth muscle hyper-proliferation. However, this type of inhibition would be problematic for the endothelium resulting in a severe reduction in the expression of anti-apoptotic proteins expression and an increase in endothelial cell apoptosis. This could subsequently promote instability of the plaque and superficial erosion of the endothelium which would result in coronary thrombus formation (Libby et al., 2006). Thus IKKβ or classical NFκB (p65) inhibitors may not be ideal for the treatment of atherosclerosis.

Prior to the beginning of the project, a number of studies showed IKKα to be involved in the regulation of inflammatory mediator production including IL-8, MCP-1 and the expression of adhesion molecules such as ICAM-1 and VCAM-1 (Denk et al., 2001; Huang et al., 2007; Gloire et al., 2007; Agarwal et al., 2005). Therefore inhibition of IKKα may be a better strategy for the treatment of atherosclerosis if endothelial cells could be targeted effectively. However, there has been very few studies which have examined the function of IKKα in endothelial cells. Studies completed early on in this project demonstrated that Adv DN-IKKα did not show any inhibitory effect on TNF-α/IL-1β induced IκB-α loss nor p65 phosphorylation, suggesting a lack of effect upon the canonical pathway and the potential to have specific roles in endothelial cell function that could be targeted. Therefore the main objectives in this thesis were to firstly determine if IKKα could be activated in endothelial cells. Secondly, to investigate the role of IKKα in the regulation of the non-canonical pathway and thirdly to determine whether this pathway could be inhibited pharmacologically.

As discussed in chapter 1, IKKα contains a nuclear localisation sequence (NLS) within kinase domain, which allows IKKα to shuffle between cytoplasm and nucleus. Early studies demonstrated that TNF-α stimulated the nuclear translocation of IKKα allowing phosphorylation of H_3 histone at ser 10 and phosphorylation of CREB binding protein (CBP) (Anest et al., 2003; Yamamoto et al., 2003). Phosphorylation at serine 10 subsequent allows acetylation of histone H3 at lysine 14 by the IKK-associated histone acetyltransferase CREB binding protein (CBP), a crucial step in modulating chromatin accessibility at NFκB-responsive promoters (Gloire et al., 2007, Huang et al., 2007, Yang et al., 2008). In addition, phosphorylation of CBP by IKKα allows a switch in the binding of CBP from p53 to p65, therefore IKKα can regulate the transcription of several p65 dependent genes (Huang et al., 2007). Whilst in this study, the nuclear translocation of IKKα was demonstrated, it was only

possible using fluorescent IKKα and translocation was weak and transient. To date no studies have convincingly demonstrated IKKα nuclear translocation in endothelial cell, therefore it is unclear if this is a feature of its activation. Time did not allow further investigating of the nuclear role of IKKα in HUVECs, but several approaches could have been considered. For example immunoprecipitation (IP) experiments could be employed to demonstrate whether IKKα is associated with CBP. Furthermore, the technique of chromatin immunoprecipitation (CHIP) could have been performed to reveal whether IKKα binds to the promoters of known p65 dependent genes such as ICAM-1 and VCAM-1 as demonstrated in other cell types. Given that inhibition of IKKα reduced both p65 NFκB reporter activity and expression of ICAM-1 and VCAM-1 in HUVECs, then the assumption would be that IKKα has a nuclear component to its range of functions in endothelial cells.

In addition to any proposed nuclear function for IKKα, substantial evidence has accumulated supporting a role for this kinase in the non-canonical pathway, which is presumed to occur within the cytosol. Two distinct pathways leading to the selective activation of RelA:p50 and RelB:p52 dimers, dependent on IKKβ or IKKα have been identified (Ghosh and Karin, 2002). Several studies have shown that only the IKKα homodimer plays a role in the regulation of the non-canonical NFκB pathway. Activation of receptors such as B cell-activating factor receptor (BAFFR), lymphotoxin β or receptor activator of NFκB (RANK), subsequently trigger p100 processing, which converts p100 to p52 and allows NFκB (Rel B/p52) to translocate into the nucleus. Indeed in this present study, it has been demonstrated that p100 processing in HUVECs is largely dependent on IKKα, results which have been confirmed by recent studies (Madge and May, 2010, Xiao et al., 2004). One of these studies suggest a negative role for IKKβ in p100 processing and the results in chapter 3 support this hypothesis. This suggests the potential for multiple points of regulation by either kinase in the context of p100 processing.

Further to this, p100 processing via IKKα can be regulated further up-stream by other intermediates. NIK is a MAP3 kinase that phosphorylates IKKα leading to p100 processing and p52 formation (Ling et al., 1998). Moreover, NIK is also responsible for ubiquitination of p100 to facilitate the processing itself (Xiao et al., 2001). Inhibition of NIK results in a reduction in NFκB reporter activity (Torrie et al., 2001). The function of NIK in endothelial cells has not been examined to date, however, in preliminary experiments, over-expression of Adv. wild type NIK resulted in an increase in p100 processing and the formation of p52 in

HUVEC, in the absence of any change in p100 protein expression (data not shown). This suggests that whilst NIK may regulate processing via IKKα, TNF-α utilising additional mechanisms. Further experiments could utilise Adv. DN-NIK to determine if p100 processing in response to TNF- α can be inhibited. Furthermore, the SU compounds which were shown to have effects on both p100 processing and protein expression could be more easily examined for inhibition of NIK induced p100 processing.

Other approaches to examining the role of IKKα in the regulation of p100 processing could involve analysis of p100 phosphorylation. Phosphorylation occurs at both serine 866 and 870 which is crucial for p100 processing (Liang et al., 2006). Given that the SU compounds had effects on both p100 processing and p100 protein expression, it would have been useful to determine whether phosphorylation was also inhibited. However, any attempts to assess p100 phosphorylation using Western blotting was unsuccessful due to interference by a number of non-specific bands making identity of the protein impossible. Other studies have been able to demonstrate p100 phosphorylation in response to different agonists such as BAFF (Claudio et al., 2002, Kayagaki et al., 2002), TNF-α (Madge et al., 2008), RANKL (Maruyama et al., 2010) and LTα1β2 (Madge et al., 2008, Mordmuller et al., 2003). In recent preliminary experiments, over-expression wild type NIK has been shown to increase p100 phosphorylation, suggesting that IKKα activated by NIK can perform this function in endothelial cells (data not shown).

Subsequent to p100 processing, Rel B/p52 translocates into the nucleus to regulate the expression of a subset of NFκB dependent genes. In some recent experiments, not included this thesis, nuclear extracts were used in Western blotting and probed for Rel B, however once again the Rel B antibody gave multiple non-specific bands. However, this and other approaches including immunofluorence, EMSA and luciferase assay would be useful for future studies to determine the impact of IKKα on the regulation of the non-canonical pathway in HUVECs. Bren and co-workers have developed selective Rel B luciferase gene constructs (1.1kb Rel B-luc and 0.25kb Rel B-luc) (Bren et al., 2001) and experiments using these constructs could determine whether DN-IKKα or SU compounds could inhibit Rel B reporter activity. CXCL12 has been identified as a Rel B/IKKα dependent gene, therefore SU compounds or DN-IKKα could inhibit not only CXCL12 gene expression but also binding of p52 to the CXCL12 promoter, assessed by CHIP. This approach could be applied to a series of IKKα/Rel B dependent genes including Blc, Sdf-1, Elc, Slc, Rxra, Irf3 and Baff (Bonizzi

et al., 2004). These genes are found mostly in lymphoid nodes which are responsible for regulation of the immune system, whether those genes are expressed in endothelial cell remains unknown.

As discussed in chapter 1, the non-canonical NFκB pathway is closely linked to angiogenesis. Angiogenesis is a process involved in blood vessel development and branching, however it is also associated with diseases such as cancers and rheumatoid arthritis. In addition, deletion of the IKKα gene in endothelial cell results in failure of microtubule formation (DeBusk et al., 2008). Moreover, IKKα also plays an important role in the regulation of angiogenic or metastatic gene expression including IL-8, VEGF and MMP-9 which are involved in colorectal cancer cell (Agarwal et al., 2005). Interestingly, in preliminary results, the SU compounds slowed down scratch induced wound healing presumably through the inhibition of endothelial cells migration (data not shown), which may indicate that IKKα can play a key role in this process. As cell migration is closely related to angiogenesis, future studies could examine whether molecular or pharmacological inhibition of IKKα could regulate microtubule formation assessed using an angiogenesis assay. In addition, it is unclear whether IKKα controls the expression of matrix metalloproteinases necessary for angiogenesis. Running a matrigel invasion assay would indicate whether IKKα can influence the degradation of the intracellular matrix. Furthermore, gene array could be employed to identify angiogenesis related genes regulated by IKKα.

As mentioned in chapter 1, IKKα and IKKβ share over 50% sequence homology within NH_2-terminal kinase domains. The problem in the development of IKKα inhibitors is that selective small molecules can also bind to the IKKβ kinase domain which limit their selectivity of the compounds. In addition, whilst the crystal structure of IKKβ has been revealed and widely used to generate IKKβ selective drugs, the equivalent structure of IKKα has not been resolved and details of the structural topography remain unknown. Although the SU compounds showed good inhibitory selectivity for IKKα over IKKβ *in vitro* and some selective inhibition of IKK function in HUVEC, higher concentrations of these compounds have potential to inhibit IKKβ, so they could not be considered for clinical use at this time. More selective and potent IKKα are required which need to be assessed in a large series of both *in vitro* and *in vivo* assays. Nevertheless, these compounds remain some of the most promising IKKα selective drugs available.

An alternative target within the non-canonical NFκB pathway is NIK. Indeed, development of a NIK inhibitor is a new approach in drug development. A series of NIK inhibitors have been recently developed and tested by Mortier and co-workers (Mortier et al., 2010), however, the IC_{50} values of the lead inhibitors, compounds 5 and 6, were found to be high at 51μM and 90μM respectively. These values indicate a very low potency and the potential of binding to other MAP3Ks such as MEKK1 or RAF, off-target effects on other pathways and issues such as cell cytotoxicity.

Another pharmacological approach may involve the disruption of protein/protein interactions. This strategy relies upon identifying defined domains of proteins that are crucial for the regulation and/or deactivation of target proteins. The site of interaction between IKKα/β and NEMO, is a hydrophobic pocket and contains a central conserved hexapeptide sequence, LDWSWL which is also termed the NBD (mentioned in section 1.3.3.7). This domain was initially predicted by means of hydropathy plots (May et al., 2000) but was more recently detailed in an IKKα/β peptide truncated NEMO co-crystal structure (Rushe et al., 2008). Development of cell-permeable peptides has become a promising approach for inhibition of NFκB. Pre-treatment with NBD peptide caused inhibition of the canonical NFκB pathway and leading to decrease in the production of IL-6, IL-12 and TNF-α and reduction of the LPS-induced maturation of dendritic cells and T cell proliferation (Tas et al., 2005). Similarly, using NBD peptide in human melanoma cultures resulted in inhibition in the NF-kB/DNA binding and also induced apoptosis via the activation of caspase 3 (Ianaro et al., 2009). This peptide may in future, provide the template for the development of small molecule inhibitors. A similar approach has yet to be tried for IKKα.

Assessment of the IKKα inhibitors implied the potential of two modes of action. Firstly inhibition of IKKα mediated processing of p100 and secondly the inhibition of IKKα mediated transcriptional activation. For the second of these actions, it is unclear if the SU compounds inhibited IKKα within the cytosol or prevented activity within the nucleus. No studies to date have confirmed that IKKα activation is required for nuclear translocation and it is interesting to note that the SU compounds are ATP binding site inhibitors. IKKα contains an NLS within the kinase domain, it is possible that some of the SU compounds may block translocation. If time had allowed studies could have been conducted to determine if the SU compounds were able to block translocation of YFP-tagged IKKα to the nucleus.

Nevertheless, the studies in this thesis demonstrate that by inhibiting IKKα, the SU compounds block the expression of inflammatory mediators including ICAM-1 and VCAM-1. More recent evidence has shown IKKα can also regulate other inflammatory genes such as IL-6, MCP-1 (Yang et al., 2008), IL-8, ICAM-1, CXCL3 (Hirata et al., 2006) and if time allowed these markers could have been examined following pre-treatment with SU compounds. Ideally, microarray studies in HUVECs could be used to undercover all IKKα dependent inflammatory genes sensitive to SU compounds including those outlined above, but also others for example, COX-2. Other arrays assessing angiogenic related genes could also be employed to gain a better overall picture of the function of IKKα in endothelial cell.

Pharmacological or molecular inhibition of the ICAM and VCAM-1 expression has been shown to attenuate the development of atherosclerosis (Fruebis et al., 1999, Pasceri et al., 2000, Patel and Kent, 1998, Zhang and Frei, 2002). IKK inhibition also reduces the release of other inflammatory mediators, thus preventing hyperproliferation of smooth muscle cells and transmigration of macrophages into the subendothelial layer. The results in this thesis suggest the potential of these effects being mediated at least partially by IKKα. Therefore drugs based on the current IKKα inhibitors designed for this project could be used to enhance the effect of statins in combination therapy without a deleterious affect on endothelial cell survival. Thus the SU compounds may have therapeutic potential in the treatment of atherosclerosis.

Nevertheless a causal link between IKKα and cardiovascular disease has not been established. Clinical data is lacking which demonstrates high levels of IKKα in atherosclerotic plaques nor additionally, a correlation between IKKα and serum markers of disease progression. Selenium is a dietary component that detoxifies lipid peroxides and has been shown to inhibit atherosclerosis more effectively than vitamin E alone (Schwenke and Behr, 1998). Zhang and co-workers have also demonstrated that selenium has inhibitory effects on ICAM-1, VCAM-1 and E-selectin expression without interfering with NFκB (p65) nuclear translocation (Zhang et al., 2002). It would be interesting to determine if selenium had any inhibitory effect on IKKα activation or transcriptional activity. A similar question could be asked of other dietary components which are protective and other pharmacological agents such as the statins. It is clear that many major studies are required to elucidate a role of IKKα in the genesis of cardiovascular disease.

In conclusion, it has been a great challenge to study the function of IKKα in HUVECs and other cell types. Recent studies suggest that the role of IKKα is a complex one and the assumption that IKKα and β may have distinctive and non-interactive roles has been continuously questioned. For example, number of recent studies show inhibition of IKKβ to result in enhanced p100 processing in HUVECs and osteoclasts (Madge and May, 2010, Ruocco et al., 2005). In addition, IKKα may also have anti-inflammatory effects in other cell types. Lawrence and co-workers have demonstrated that IKKα can inhibit p65 dependent gene expression including MIP-1β, MIP-2, TNF-α and ICAM-1 in macrophages (Lawrence et al., 2005). This effect has been shown mediated by a negative feedback effect on the canonical NFκB pathway. For instance, IKKα phosphorylates the protein inhibitor of activated STAT1 (PIAS-1), which inhibits p65 activity (Liu et al., 2007). Furthermore, a recent study showed that IKKα directly phosphorylates the regulatory molecule TAX1BP1, which allows the complex containing TAX1BP1, Ltch and RNF11 to interact with A20 to form the A20 ubiquitin-editing complex which subsequently inhibits p65 activity (Shembade et al., 2011). These and other types of study could be performed in HUVECs to allow better understanding of the role of this important kinase in endothelial cell function in both health and disease.

CHAPTER 6

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