Investigating the role of Pellino1, an E3 ubiquitin ligase, in modulating signalling pathways controlling the inflammatory response

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Summary

Pellino1 was identified as an IRAK binding partner and is involved in Lysine-63 polyubiquitination of IRAK 1/4 and RIP1, which are key mediators of the TLR and IL-1R signalling pathways. As such, these pathways are implicated in responses to bacterial and viral infections and common respiratory viruses, such as rhinovirus, are a major cause of asthma exacerbations.

The functional importance of Pellino proteins in regulating immune responses and their role in airway inflammatory diseases is yet to be elucidated. We hypothesised that Pellino1 would play a critical role in regulating the inflammatory response in the lung airway epithelium.

Expression and regulation of the Pellino family was investigated at the transcriptional level. Pellino1 was knocked down in the human bronchial airway epithelial cell line BEAS-2B and PBECs using targeted siRNA.

Pellino1 is expressed in BEAS-2B and PBECs. Pellino1 knockdown led to reduced IL-8 generation in response to IL-1β and a viral mimic poly(I:C) stimulation in BEAS-2B, however RANTES production was unchanged. In contrast to the BEAS-2B, the PBECs exhibited preserved IL-1 signalling. However, these cells also showed a reduction in IL-8 production in response to both poly(I:C) stimulation and RV1B infection. Pellino1 knockdown had no effect on RANTES production. Knockdown of a putative Pellino1 target, RIP1, in PBECs leads to the significant increase in IL-8 generation in response to poly(I:C), suggesting that it may act as a negative regulator of the TLR3 signalling pathway in these cells. PBECs may utilise the non-canonical NF-κB signalling pathway in response to poly(I:C) stimulation.

Pellino1 knockdown leads to reduced IL-8 production in response to a viral mimic poly(I:C) in lung airway epithelial cells and the natural pathogen RV1B in PBECs. These data indicate that Pellino1 regulates proinflammatory responses in airway epithelium and may be a feasible target to downregulate neutrophilic airway inflammation whilst retaining antiviral immunity, which would be highly beneficial in the treatment of chronic airway inflammatory disorders such as asthma and COPD.

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Publications and Conference Abstracts

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Stokes, C.A., Ismail, S., Dick, E.P., **Bennett, J.A.,** Johnston, S.L., Edwards, M.R., Sabroe, I., Parker, L.C. (2011) Role of Interleukin-1 and MyD88-Dependent Signaling in Rhinovirus Infection. *Journal of Virology.* 85 (15), 7912-7921.

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Abbreviations

AcP	accessory protein
AP-1	activator protein 1
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
bp	base pair
BPI	bactericidal permeability-increasing
bZIP	basic leucine zipper domain
CARDs	caspase recruitment domains
cDNA	complementary DNA
COPD	chronic obstructive pulmonary disease
CPE	cytopathic effect
DAMP	damage associated molecular pattern
DD	death domain
DMEM	Dulbecco's Modified Eagle Medium
dsRNA	double-stranded RNA
ECL	enhanced chemiluminescence
ERK	extracellular signal-regulated kinases
FAM	6-carboxyfluorescein
FCS	foetal calf serum
FHA	forkhead-associated domain
GRR	glycine-rich region
HBSS	Hank’s buffered salt solutions
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
ICAM-1	intercellular Adhesion Molecule 1
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase
IL-1	interleukin 1
IL-1R	interleukin 1 receptor
IL-6	interleukin 6
IL-8	interleukin 8
IP-10	interferon gamma-induced protein 10

IRF	interferon regulatory factor
IRAK	IL-1R associated kinase
ISG	interferon-stimulated gene
JAK	janus kinase
JNK	c-Jun N-terminal kinases
Kd	dissociation constant
KD	kinase domain
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MDA5	Melanoma Differentiation-Associated Gene 5
MEFs	mouse embryonic fibroblasts
MMP	metalloproteinases
M.O.I	multiplicity of infection
MPO	myeloperoxidase
MyD88	myeloid differentiation primary response gene 88
NDV	Newcastle disease virus
NE	neutrophil elastase
NEMO	NF-κB-essential modulator
NF-κB	nuclear factor kappa B
NIH	National Institutes of Health
NIK	NF-κB binding kinase
NLS	nuclear location sequence
PAMP	pathogen associated molecular pattern
PBECs	primary bronchial epithelial cells
PBMCs	peripheral blood mononuclear cells
PLA_2	Phospholipases A2
PMSF	phenylmethanesulfonylfluoride
Poly(I:C)	polyinosinic:polycytidylic acid
PPP	platelet poor plasma
qPCR	quantitative PCR
RANTES	regulated upon activation in normal T-cells, expressed and secreted
RV	rhinovirus
RNA	ribonucleic acid
RHD	rel homology domain

RIG-I	retinoic acid-inducible gene-I
RING	really interesting new gene
RIP	receptor interacting protein
rpm	repetitions per minute
RSV	respiratory syncytial virus
RT-PCR	reverse transcription polymerase chain reaction
SAPK	stress-activated protein kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
SeV	Sendai virus
shRNA	short hairpin RNA
S.O.C	super optimal broth with catabolite repression
ssRNA	single-stranded RNA
STAT	signal transducer and activator of transcription
TAB	TAK1 binding protein
TACE	TNFα converting enzyme
TAD	transactivating domain
TAK1	transforming growth factor β–activated kinase 1
TAMRA	tetramethylrhodamine
TBK1	TANK binding kinase 1
TEMED	tetramethylethylenediamine
TIMP	tissue inhibitors of metalloproteinases
TIR	TLR/IL-1R
TLR	toll-like receptor
TNFα	tumour necrosis factor alpha
TRAF6	TNF receptor associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon-β
TWEAK	TNF-like weak inducer of apoptosis
Ubc	ubiquitin-carrier/conjugating protein
UTR	untranslated region
VSV	vesicular stomatitis virus
vLDLR	very low density lipoprotein receptor
WHO	world health organisation
w/v	weight/volume ratio

1 Chapter 1 - Introduction

1.1 Innate immunity and inflammation: an overview

Pathogens are biological entities, usually bacteria, fungi or viruses that cause disease in another organism. Hosts and pathogen evolve together in an antagonistic manner, each trying to overcome the defence mechanisms of the other. The collective term for the mammalian defence mechanisms is the immune system, which is a complex network of specialist cells that can distinguish between self and non-self (foreign material) and act to detect pathogens, prevent infection and resolve sterile tissue damage.

The immune system can be broadly split into two components, the innate and adaptive immune system. The former provides the first form of defence against pathogens and is characterised by immediate reaction to foreign material or endogenous danger signals that induce the inflammatory response. The acute inflammatory response plays an important role in activating and recruiting cells involved in the immune and wound-healing response. Inflammation is characterised by erythema (rubor), oedema (tumor) and capillary dilation allowing for white blood cell transmigration. Despite the crucial role of inflammation in host defence, it is also the root-cause of many chronic diseases such as asthma, chronic obstructive pulmonary disease (COPD) (reviewed in (O'Byrne P and Postma 1999)) and arthritis.

Neutrophils and macrophages are principal leukocytes that contribute to the innate immune response. Two thirds of the peripheral blood leukocyte population are neutrophils (Yamashiro, Kamohara et al. 2001), which are short-lived (half life of 6-8 hours), fast moving (*in vivo*) polymorphonuclear granulocytes, whose main function is to engulf and degrade bacteria (Segal 2005). Neutrophils are normally fully differentiated once they are released from the bone marrow (Akgul, Moulding et al. 2001) and in the absence of "danger signals" (Matzinger 1994) such as proinflammatory cytokines, aged neutrophils spontaneously undergo apoptosis (Savill, Henson et al. 1989) and are engulfed and cleared by macrophages. Apoptosis is a tightly controlled process, which is anti-inflammatory by both preventing tissue damage and promoting neutrophil clearance by macrophages (Akgul, Moulding et al. 2001). Neutrophils are rapidly recruited to the site of infection, where they engulf extracellular bacteria and debris into the phagosome, which fuses with the lysosome to form the phagolysosome. In the phagolysosome, reactive oxygen species (O_2^-, H_2O_2 etc.) are released during a respiratory burst where electrons are passed from the NADPH oxidase complex to oxygen (Segal and Abo 1993). Neutrophils also contain many

other mediators that are involved in the degradation of pathogens and extracellular matrix proteins including myeloperoxidase (MPO), elastase, cathepsin G, proteinase-3, defensins, bactericidal permeability-increasing (BPI) protein and azurocidin (Campanelli, Detmers et al. 1990). These potentially harmful proteases are encapsulated within granules located in the neutrophil cytoplasm to restrict damage to the host and are only released upon activation of the neutrophil by a process named degranulation, which usually occurs in response to the increase in intracellular calcium (Niessen, Kuijpers et al. 1991).

Mononuclear cells differentiate in the bone marrow into pro-monocytes, which are released into the peripheral blood as monocytes (10-18μm in diameter). Further differentiation occurs as monocytes transmigrate into the tissues and become larger (21μm diameter) long-lived (months) macrophages. Mononuclear cells are prominent phagocytes involved the innate immune response, but differ from neutrophils in that they are synthetic cells that play a major role in producing the signalling molecules of the immune system, cytokines. Cytokines are small (8-20 kDa) proteins or glycoproteins that bind to cell-surface receptors that activate intracellular signalling pathways that usually lead to upregulation of target genes. A potent pro-inflammatory cytokine produced by macrophages is interleukin 1β (IL-1β) which causes vasodilation (swelling), leaky blood vessels (redness), and fever. Macrophages also interact with cells of the adaptive immune system, T- and B-cells, to initiate and influence the adaptive immune response by acting as antigen-presenting cells.

Airway epithelial cells are the first cell-type to be impacted by inhaled environmental factors such as bacterial or viral pathogens, allergens, pollutants and inhaled medications for airway diseases. The classical view of the airway epithelium was that it acted as a passive barrier between the host and the environment. More recently, however, it has been shown that the airway epithelium plays a more dynamic role in the activation and regulation of the innate immune system. Airway epithelial cells can produce a plethora of cytokines including IL-6 and IL-8 (Cromwell, Hamid et al. 1992; Subauste, Jacoby et al. 1995; Tomee, Wierenga et al. 1997; King, Brennan et al. 1998) in response to various pro-inflammatory stimuli; and interferons (IFNs) (Type I and III) and interferon-stimulated genes (ISGs) in response to viral stimuli (Tudhope, Catley et al. 2007). Airway epithelial cells do not produce much IL-1β (Proud and Leigh 2011), however IL-1β has been shown to be an important mediator in the co-operation between the networks of leukocytes and epithelial cells that are present in the airway during infection (Morris, Parker et al. 2006).

Pathogen recognition is accomplished by receptors that bind to either endogenous danger signals such as IL-1, or molecules that highlight a microbial infection, known as pathogen-associated molecular patterns (PAMPs). Damage-associated molecular patterns (DAMPs) are molecules that are released from necrotic cells and act as danger signals to promote an inflammatory response. A key DAMP is High Motility Group Box 1 (HMBG1), which is a nuclear protein that activates the immune response under stressed conditions.

PAMPs and DAMPs are recognised by pattern-recognition receptors such as Toll-like receptors. Toll was first discovered in *Drosophila melanogaster* as a protein responsible for dorsoventral orientation in the *Drosophila* embryo, but it was observed that $Toll^{-/-}$ mutants were highly susceptible to fungal infections (Lemaitre, Nicolas et al. 1996), suggesting its involvement in the immune system. Subsequently, a family of mammalian Toll-like receptors were found to be an essential part of the innate immune system. Signalling initiated by TLRs and the IL-1 receptor is an intricate network of pathways that contain a complex web of adaptor proteins leading to the activation of effector molecules such as kinases and transcription factors. This project has focused on the role of an E3-ubiquitin ligase, Pellino1 and its effect on the regulation of the signalling networks controlling inflammation.

1.2 Chronic inflammatory diseases of the lung

Chronic respiratory diseases are diseases of the lung and airways that are characterised by chronic airway inflammation and increased airway hyperresponsiveness to a range of agonists including viral or bacterial infections, cigarette smoke, allergens and pollution. Respiratory disorders are prevalent throughout the world; where according to the World Health Organisation (WHO) estimates (2004), currently 64 million people suffer from chronic obstructive pulmonary disease (COPD) and 235 million people have asthma.

1.2.1 Chronic obstructive pulmonary disorder

Chronic obstructive pulmonary disorder (COPD) is a term used to describe a range of related processes that lead to a clinical phenotype of limitation in lung airflow that is poorly reversible. The most common symptoms for COPD include breathlessness, excessive sputum production and chronic cough. COPD is a major and increasing world healthcare burden and causes approximately 25,000 deaths in the UK per year and is the fourth most common cause of death in the USA and according to research carried out by WHO in 2001, this figure has continued to rise over the past 30 years.

Inflammation is intrinsically linked to the progression of COPD, which is characterised by: chronic obstructive bronchitis with fibrosis and obstruction of small airways; emphysema with enlargement of airspaces and destruction of lung parenchyma; reduction in the elasticity of the lung; and occlusion of the small airways. Bronchial biopsies, small airways and parenchyma from COPD patients show increased leukocyte infiltration of monocytes and neutrophils, and this alongside airway remodelling through fibrosis is thought to be the major cause of progressive, irreversible airway narrowing (Hogg, Chu et al. 2004). Neutrophils produce cytotoxic agents that include serine proteases including neutrophil elastase (NE), cathepsin G, and proteinase-3, as well as metalloproteinases (MMP)-8 and MMP-9, which could contribute to the breakdown of the alveolar wall (emphysema). Increased numbers of neutrophils have been found in the bronchoalveolar lavage (BAL) fluid and sputum of COPD patients (Lacoste, Bousquet et al. 1993; Keatings, Collins et al. 1996).

Alveolar macrophages have been shown to play an important role in COPD, as they produce a cohort of proteases that contribute to emphysema, including MMP-2, MMP-9, MMP-12, cathepsins K, L, and S, and neutrophil elastase taken up from neutrophils. There has been shown to be a 5- to 10-fold increase in macrophages in the airways, BAL fluid, sputum and lung parenchyma in COPD patients compared to healthy volunteers (Retamales, Elliott et al. 2001). These macrophages are involved in the production of increased concentrations of proinflammatory cytokines such as IL-1β, TNFα and IL-6. These cytokines are regulated by the transcription factor NF-κB, which has been shown to be activated in alveolar macrophages in COPD patients, especially during exacerbations (Caramori, Romagnoli et al. 2003).

There is currently no cure for COPD so current therapies focus on alleviating symptoms, slowing the progression of the disease and increasing the patients' tolerance to exercise. Treatments include bronchodilators, inhaled glucocorticosteroids and oxygen therapy in severe cases of COPD. Research into the molecular mechanisms causing COPD will be invaluable in developing new treatments for this disease.

1.2.2 Asthma

Asthma is a chronic airway inflammatory disorder, which is associated with increased airway hyperresponsiveness. Currently 5.4 million people are receiving treatment for asthma in the UK, which equates to 1 in 12 adults and 1 in 11 children (source: AsthmaUK). There is no cure for asthma and so the symptoms of wheeze, cough, breathlessness and

chest tightness are alleviated using "reliever inhalers" that contain short acting β_2-agonist that act by relaxing the smooth muscle around the narrowed airway. These are used in conjunction with inhaled corticosteroids to suppress airway inflammation. Asthma presents a major healthcare and economic burden and cases of the disease have risen in both adults and children over the last 40 years (Upton, McConnachie et al. 2000; Pearce and Douwes 2006; Wilson, Adams et al. 2006).

Asthma exacerbations are a severe onset of symptoms, which can be caused by numerous factors including exposure to allergens such as pollen, dust mites or animal dander (Gelber, Seltzer et al. 1993; Custovic, Taggart et al. 1996; Djukanovic, Feather et al. 1996), air pollution (Kesten, Szalai et al. 1995; van der Zee, Hoek et al. 1999), cigarette smoke (Chilmonczyk, Salmun et al. 1993), exposure to certain chemicals (Mapp, Boschetto et al. 2005), exercise (Suman, Beck et al. 1999), non-steroidal anti-inflammatory drugs (Szczeklik and Stevenson 1999), and respiratory infections. Respiratory tract infections have been shown to be a major cause of asthma exacerbations (Nicholson, Kent et al. 1993; Johnston, Pattemore et al. 1995; Teichtahl, Buckmaster et al. 1997; Atmar, Guy et al. 1998), with viral infections being the most prominent type of infection (Nicholson, Kent et al. 1993; Atmar, Guy et al. 1998; Papadopoulos and Johnston 1998), however bacterial pathogens have also been shown to play a role in asthma exacerbation (Cunningham, Johnston et al. 1998; Lieberman, Printz et al. 2003). Infection with the common respiratory virus rhinovirus is most frequently associated with asthma exacerbations in both adults (Nicholson, Kent et al. 1993) and children (Johnston, Pattemore et al. 1995) (**see section 1.3.1**).

Factors that lead to asthma exacerbations can also act in a synergistic manner to cause a more severe asthma exacerbation. Respiratory tract that has been sensitised by allergen exposure followed by viral infection of the respiratory tract has been shown to increase the risk of hospitalisation due to acute asthma in both adults and children (Green, Custovic et al. 2002).

Asthma exacerbations are currently treated with inhaled glucocorticosteroids as these steroids produce an anti-inflammatory effect by reducing the transcription of proinflammatory genes (Barnes 1998), perhaps by binding directly to, and inhibiting, NF-κB (Ray and Prefontaine 1994; Adcock, Brown et al. 1995; Caldenhoven, Liden et al. 1995). Also, these steroids are involved in upregulating anti-inflammatory proteins such as IL-1 receptor antagonist (IL-1ra) (Levine, Benfield et al. 1996; Sousa, Lane et al. 1996). However, occurrences of asthma exacerbations in patients with persistent asthma are only reduced

by approximately 45% with this treatment (Pauwels, Pedersen et al. 2003) and asthmatic patients that had been experimentally infected with rhinovirus did not exhibit a significant reduction in airway inflammation upon treatment with inhaled glucocorticosteroids (Grunberg, Sharon et al. 2001), despite beneficial results found *in vitro* (Papi, Papadopoulos et al. 2000; Edwards, Johnson et al. 2006; Edwards, Haas et al. 2007). Combination therapy which combines steroid treatment with that of long acting β_2-agonists have shown some promise in the reduction of asthma exacerbations in patients (O'Byrne, Bisgaard et al. 2005; Lundborg, Wille et al. 2006), however does not eradicate exacerbations and the effect of combination therapy in response to rhinoviral-induced asthma exacerbations has yet to be investigated.

Asthma is becoming more prevalent in the world's population and so there is a demand for research into the molecular mechanisms that underpin such inflammatory diseases. This research, along with research into the many factors that lead to exacerbations such as rhinoviral infection, will aid the progression of the development of new and more tailored treatment of asthma. One of the main ways in which the immune system detects pathogens is by the evolutionary conserved TLRs binding to PAMPs. Stimulation of these receptors leads to the activation of intracellular signalling pathways that control the production of antimicrobial or inflammatory agents. The key mediators of TLR signalling pathway are outlined in **section 1.7 – 1.9**.

1.3 Rhinovirus

Human rhinoviruses (RV) are a major cause of the common cold in humans, which is one of the most frequent infectious diseases in humans. RV infection in healthy individuals usually causes no lasting harmful effects, however RV infections are a major cause of asthma (Johnston, Pattemore et al. 1995; Johnston, Pattemore et al. 1996; Gern and Busse 1999) and COPD exacerbations (Seemungal, Harper-Owen et al. 2000), pneumonia and bronchiolitis in young children (Renwick, Schweiger et al. 2007). Despite RV being a simple virus with a small RNA genome, they are one of most successful human pathogens (Pitkaranta and Hayden 1998; Whitton, Cornell et al. 2005), which is mainly due to the constant evolution of the virus that is accomplished by their error-prone RNA polymerase inserting approximately 10^{-3} and 10^{-4} errors/nucleotide/cycle of replication (Drake 1999; Harvala and Simmonds 2009).

RVs are the largest subgroup of the *Picornaviridae* family of viruses of which 102 serotypes have been discovered to date. RVs are small (25-30 nm diameter), non-enveloped viruses

with a single-stranded, positive sense RNA genome that is encapsulated within an icosahedral capsid. The most exposed regions of the capsid are hyper-variable and give rise to the antigenic diversity between the RV serotypes (Rossmann and Palmenberg 1988).

The genome is approximately 7.5 kb in length containing a 5' untranslated region (UTR) of approximately 620 bp, which is bound by a covalently linked virus protein (VPg), and a 3' UTR (~50 bp) connected to a 3' Poly-A tail (Johnston, Bardin et al. 1993). The VPg protein is lost upon entry into the host cell and the 5' UTR forms a cloverleaf structure through internal base pairing that allows ribosome binding for CAP-independent translation of the viral genome (Belsham and Sonenberg 1996). The viral RNA encodes a single polypeptide that is subject to post-translational processing by viral-encoded proteases to produce mature viral proteins. The viral polypeptide is divided up in to three regions, P1, P2 and P3. P1 encodes four structural proteins, VP1-4, of which 60 copies of each are used to create the icosahedral capsid. P2 and P3 regions encode non-structural proteins, many of which are involved in viral replication including two viral proteases, VPg and RNA-dependent RNA polymerase (Kirchberger, Majdic et al. 2007).

RVs are divided into three groups mainly based upon the receptor they utilise for internalisation into the host cell. The major group, of which approximately 90% of RVs belong, enter host cells by binding to intercellular adhesion molecule 1 (ICAM-1) (Greve, Davis et al. 1989; Staunton, Merluzzi et al. 1989) and the minor group utilise the very low density lipoprotein receptor (vLDLR) for host cell entry (Marlovits, Abrahamsberg et al. 1998; Marlovits, Zechmeister et al. 1998). Major and minor group RVs are taken up into the cell by endocytosis and low pH is required for the efficient un-coating and release of viral RNA into the cytoplasm (Bayer, Schober et al. 1998; Suzuki, Yamaya et al. 2001; Nurani, Lindqvist et al. 2003). More recently, a distinct group of RVs, known as HRV-C, has been identified through substantial genetic divergence from other classical species within the genus. However, due to the inability to culture these strains *in vitro*, the receptor that is utilised for internalisation by HRV-C is currently unknown (Arden and Mackay 2010).

Upon RV infection, host cell transcription, translation and modification of intracellular membrane structures are all inhibited (Belsham and Sonenberg 1996) and the RV single stranded RNA genome binds to ribosomes in the cytoplasm for translation of the viral polypeptide. The RV genome can be reverse transcribed by the viral RNA-dependent RNA polymerase to create a template for the synthesis of new viral progeny. The RV is very efficient at taking over host cell machinery and such within 6 hours up to 100,000 virions

can be produced from a single infection, which leads to host cell rounding and subsequent lysis releasing viral progeny into the respiratory tract where it can infect surrounding epithelia (Belsham and Sonenberg 1996).

1.3.1 Rhinovirus involvement in asthma exacerbations

RV infections of the upper respiratory tract are usually harmless, self-limiting infections that provide no lasting harmful effects. RVs primarily infect the upper respiratory tract, however there is increasing evidence to suggest that they are also capable of replicating in the lower respiratory tract (Kirchberger, Majdic et al. 2007). In normal respiratory tract, RV can only replicate in approximately 10% of epithelial cells and produces very little cytopathic effect (Heikkinen and Jarvinen 2003), however RV infection does lead to the increase in proinflammatory mediators including histamine (Welliver, Wong et al. 1981), IL-1, IL-6, IL-8/CXCL8, TNFα (Noah, Henderson et al. 1995), RANTES/CCL5 (Schroth, Grimm et al. 1999) and IP-10/CXCL10 (Spurrell, Wiehler et al. 2005). Increasing concentrations of IL-6 and IL-8 correlate to increasing severity of symptoms in RV infection (Zhu, Tang et al. 1996; Turner, Weingand et al. 1998). RV infection leads to an inflammatory response by the host (Kirchberger, Majdic et al. 2007) and therefore may be linked to asthma exacerbations due to the hyperresponsiveness of the asthmatic airway.

Recent studies have used RT-PCR to detect virus and found that viral infections are commonly associated with asthma exacerbations. Several different viruses including enteroviruses, influenza viruses, respiratory syncytial virus (RSV), adenoviruses, coronaviruses and parainfluenza viruses have all been associated with asthma exacerbation in children (Freymuth, Vabret et al. 1999) and adults (Nicholson, Kent et al. 1993; Atmar, Guy et al. 1998; Grissell, Powell et al. 2005), with RV infection being identified as the most common trigger.

In more severe cases, RV can be detected in the majority of children over the age of three that have been hospitalised due to asthma exacerbation (Freymuth, Vabret et al. 1999; Heymann, Carper et al. 2004; Kling, Donninger et al. 2005). This includes a study of 179 asthmatic children hospitalised for asthma exacerbation which detected RV in 79% of cases, which was significantly greater than the detection levels in non-symptomatic asthmatic (17%) and non-ambulatory children with respiratory illness (52%) (Rawlinson, Waliuzzaman et al. 2003). Respiratory viral infections were also detectable in a high number (55-78%) (Atmar, Guy et al. 1998; Wark, Johnston et al. 2002; Grissell, Powell et al.

2005) of adults attending the emergency department with acute asthma exacerbation and a majority of these infections were RV (Atmar, Guy et al. 1998; Grissell, Powell et al. 2005).

As noted previously, RV infection of the respiratory tract leads to the production of proinflammatory cytokines including IL-8. IL-8 is a potent chemoattractant for neutrophils and in contrast to the allergen-mediated asthma exacerbation which is associated with the infiltration of eosinophils, viral-induced exacerbations are associated with neutrophilia (Fahy, Kim et al. 1995). This neutrophilia is detrimental towards the patient as the inflammatory environment of the asthmatic airways leads to the prolonged survival of the neutrophils and thus prolonged production of harmful cytotoxic agents and proteases. It has also been suggested that IL-8 is the major chemokine that creates a neutrophilic environment in asthmatic airways (Fahy, Kim et al. 1995).

However, the molecular mechanism by which RV causes asthma exacerbations is not yet fully understood. Factors other than increased numbers of neutrophils can be responsible for asthma exacerbation, since neutrophil numbers did not differ between control and asthmatic patients with acute RV infection in a recent study (Kirchberger, Majdic et al. 2007). Research into the regulation of viral signalling pathways is highly important in the design of new therapies for asthma and associated inflammatory disorders.

1.4 Toll-like receptors

Toll-like receptors (TLRs) are highly conserved (sequence homology reaching back to Toll receptors in *Drosophila melanogaster*), pattern-recognition receptors that are integral to innate immune defences since they bind to PAMPs and DAMPs. Over ten TLRs have been found in the mammalian genome, all of which bind to PAMPs (Akira, Takeda et al. 2001) (an overview can be found in **Table 1.1**)**.** TLRs are type I transmembrane receptors that are characterised by an extracellular leucine-rich region containing the $L(X_2)LXL(X_2)NXL(X_2)L(x_7)L(X_2)$ consensus sequence that is important in ligand binding and signal transduction (Kobe and Deisenhofer 1994).

Most cells express at least one TLR and proinflammatory cytokines (TNF, IFNs) typically cause upregulation of TLRs at the cell surface (Muzio, Bosisio et al. 2000). The functional importance of TLRs was demonstrated by initial cell-line experiments that linked TLRs to the innate immune system, followed by the creation of $TLR^{-/-}$ knock-out mice, which demonstrated an inability to respond to certain pathogens (Akira and Takeda 2004). Even single nucleotide polymorphisms in TLRs can lead to an increased susceptibility to certain

diseases, for example the naturally occurring Thr399Ile in TLR4 leads to a predisposition to severe malaria in African children (Mockenhaupt, Cramer et al. 2006), and Arg753Gln in TLR2 manifests an increased susceptibility to tuberculosis (Pandey and Agrawal 2006). Thus TLR signalling is tightly regulated and the disruption of regulation can lead to impaired immune responses and disease.

1.5 Interleukin 1 Receptors

IL-1 is a potent pro-inflammatory cytokine (**see section 1.11.1**). The IL-1 receptor belongs to the immunoglobulin class of receptors that have 3 extracellular immunoglobulin-like domains at the N-terminus, where ligand is bound. There are two types of the IL-1 receptor, IL-1RI and IL-1RII.

IL-1RI is the 80kDa "signalling receptor" that binds IL-1 with high affinity (5-500 pM Kd) and transduces the signal that leads to the production of proinflammatory cytokines. (Fitzgerald and O'Neill 2000) However, signal transduction can only occur in the presence of the IL-1 accessory protein (AcP). AcP has no affinity for IL-1 itself, but as the ligand binds, IL-1RI recruits AcP to form a high affinity heterotrimeric signalling complex (Greenfeder, Nunes et al. 1995).

As mentioned previously, IL-1 is a potent pro-inflammatory cytokine. Another way in which IL-1 potency is attenuated is by the IL-1RII, which is a 67-kDa "decoy receptor" (Bourke, Cassetti et al. 2003) that has a high avidity for IL-1β and is able to form the heterotrimeric signalling complex but does not mediate any cellular responses (Lang, Knop et al. 1998). IL-1RII is structurally related to IL-1RI, except IL-1RII has a much shorter cytoplasmic tail (19 and 215 amino acids, respectively). IL-1RII is mainly found on B-cells, macrophages and peripheral blood neutrophils and acts a sink for IL-1, therefore down-regulating the effects of this potent pro-inflammatory molecule.

1.6 TIR domain

TLR and IL-1R both function to detect danger signals and initiate innate immune responses. This connection is shown clearly by the use of common intracellular signalling components, beginning with homologous cytoplasmic receptor domains. The TLR/IL-1R (TIR) domain is a stretch of ~200 amino acids in the intracellular region of the TLR/IL-1R that are arranged in 3 conserved domains, named boxes (Akira 2003). Box1 is the signature sequence of the family and box2 and box3 contain sequences critical for signalling (Akira 2003). X-ray crystallography exposed a core structural element centred around box2 named the BB-loop, which contains a critical proline or arginine residue at the tip of the loop that is involved in forming a contact with downstream signalling components (**Figure 1.1**) (Khan, Brint et al. 2004). The most common inflammatory route utilises the adaptor molecule MyD88 and hence is known as the MyD88 dependent pathway, the major components of which are discussed in the following sections.

Receptor	Localisation	Ligands
TLR1	Cell surface	Bacterial lipoproteins – triacyl lipoprotein (with TLR2)
TLR2	Cell surface	Bacterial cell wall components – lipoproteins, lipoteichoic acid. Zymosan (fungi).
TLR3	Intracellular	Viral double stranded RNA
TLR4	Cell surface	Lipopolysaccharide (Gram-negative bacteria), taxol (antitumour agent in humans) and F protein (syncytial virus)
TLR5	Cell surface	Flagellin
TLR6	Cell surface	Diacyl lipoproteins (with TLR2)
TLR7, TLR8	Intracellular	Guanosine and uridine from single chain RNA (TLR8). Loxoribine (a guanosine analogue) TLR7. Imidazoquinoline (antiviral compound). Bacterial DNA and viral RNA
TLR9	Intracellular	Bacterial and viral CpG DNA motifs
TLR10	Cell surface	Unknown, although has been shown to dimerise with TLR1 and 2 (Hasan, Chaffois et al. 2005)

Table 1.1: TLRs and their ligands

Table 1 lists the pathogen associated molecular patterns (PAMPs) that act as TLR agonists and the cellular localisation of each TLR (Crespo-Lessmann, Juarez-Rubio et al. 2010).

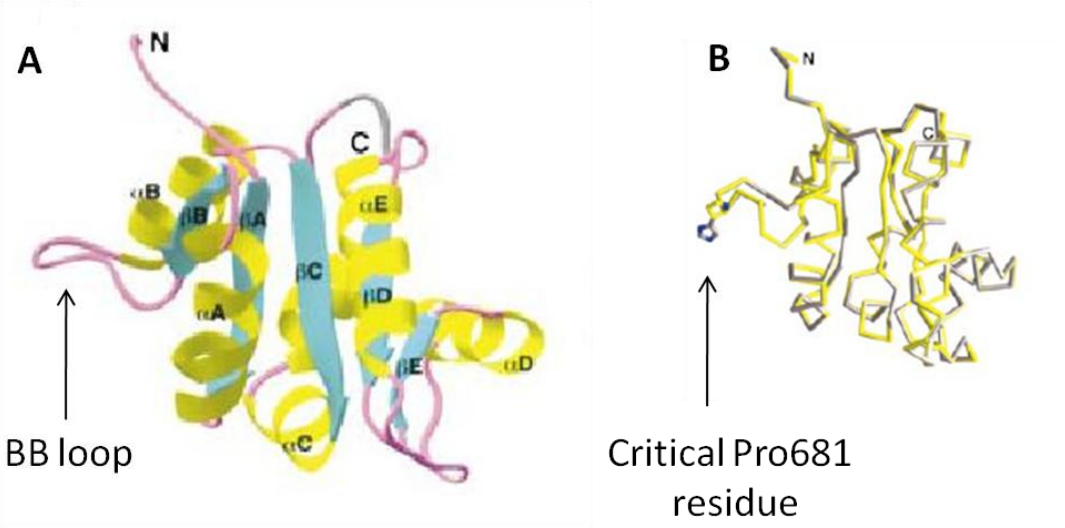


Figure 1.1: Crystal structure of the TIR domain

The crystal structure of the TIR domain is represented as a ribbon diagram of alpha helices making up the TIR domain showing the BB loop structural domain that is critical for downstream signalling of TLR/IL-1R (**A**) and a stick diagram of TLR2 TIR domain showing the position of the critical proline residue necessary for contacting downstream signalling components (adapted from (Xu, Tao et al. 2000)).

1.7 Key mediators of the MyD88-dependant TIR signalling pathway

1.7.1 MyD88

MyD88 was first discovered as a myeloid differentiation response gene that was induced in M1 myeloblastic leukaemia cells upon stimulation with IL-6 and caused growth arrest and terminal differentiation into macrophages (Lord, Hoffman-Liebermann et al. 1990). Sequence analysis uncovered a C-terminal TIR domain that mediates homophylic interactions with other TIR domains, and is recruited to activated TLR/IL-1R receptors (Xu, Tao et al. 2000). The N-terminus contains a death-domain (DD), so called as they were first discovered in proteins linked to apoptosis but now they are known to mediate protein-protein interactions (Feinstein, Kimchi et al. 1995), thus making MyD88 an adaptor molecule that links the receptor to downstream signalling components. Following receptor activation, MyD88 functions to recruit IL-1R-associated kinases (IRAKs) (Wesche, Henzel et al. 1997) (see **Figure 1.2**).

1.7.2 IRAKs

IRAKs are a family of four kinases, IRAK-1, IRAK-2, IRAK-4 and IRAK-M, which are all separate gene products that are scattered around the genome. *Irak-1* has been mapped to human chromosome Xq28 and produces a 90kDa protein (Thomas, Allen et al. 1999), *Irak-2* is found at 3p25 and transcribes a 65kDa protein (Janssens and Beyaert 2003), both of which are ubiquitously expressed (Cao, Henzel et al. 1996; Muzio, Ni et al. 1997). *Irak-M,* predominantly expressed in peripheral blood leukocytes, is located at 12q14 and produces a 68kDa protein, and finally *Irak-4* is found at position 12p11 and encodes a 52kDa protein that is expressed at low levels in many tissues (Li, Strelow et al. 2002). Despite the four IRAK molecules being separate gene products, they share a similar amino acid sequence (Janssens and Beyaert 2003).

All IRAKs are made up of the same functional domains. At the N-terminal there is a ~90 amino acid DD that is required for protein-protein interactions. The central region is devoted to a kinase domain (KD) that has conventional characteristics of other serine/threonine kinases and contains 12 subdomains (Johnson, Noble et al. 1996). All IRAKs contain an ATP-binding site, but interestingly IRAK-2 and IRAK-M contain a mutation that renders their KD inactive (Wesche, Gao et al. 1999).

IRAK-2 has been implicated in TLR2/4 specific signalling pathways but does not bind IRAK-1 (Fitzgerald, Palsson-McDermott et al. 2001) and has been shown to be essential for TRAF6 activation (Keating, Maloney et al. 2007). Interestingly, IRAK-M has been shown to be a negative regulator of TIR signalling, as IRAK-$M^{-/-}$ macrophages produce substantially more cytokine in response to an array of TLR/IL-1R agonists (Kobayashi, Hernandez et al. 2002). The mechanism remains elusive, but it has been suggested that IRAK-M traps IRAK-1 and IRAK-4 at the receptor complex in the unphosphorylated state (Janssens and Beyaert 2003).

IRAK-1 kinase activity is strongly augmented by IL-1; however it is not essential for IL-1 signal transduction (Knop and Martin 1999) unlike the vital KD of IRAK4 (Burns, Janssens et al. 2003). IRAK-1 and IRAK-4 are recruited to the receptor complex by binding to MyD88 DD, and IRAK-4 phosphorylates IRAK-1, which is thought to activate IRAK-1 kinase domain that leads to autophosphorylation of IRAK-1 (Janssens and Beyaert 2003). Phosphorylation deceases the affinity of IRAK-1 for MyD88, whilst increasing its affinity for tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6), the next step in the pathway (Janssens and Beyaert 2003) (see **Figure 1.2**).

1.7.3 TRAF6

TRAF6 is a member of an evolutionary conserved family of TNF receptor-associated adaptor proteins that is recruited to the TIR complex via the TRAF-C domain that mediates interaction with the TRAF6 binding motif (Pro-X-Glu-X-X-aromatic/acidic residue) found in the C-terminal stretch of IRAK-1 (Ye, Arron et al. 2002). The N-terminus contains the effector domains of zinc-fingers and RING domains (Baud, Liu et al. 1999) that are activated upon binding to IRAK-1. This allows the dissociation of IRAK-1: TRAF6 from the receptor complex and subsequent binding of another preformed membrane-bound signalling complex made up of transforming growth factor β (TGF-β)-activated kinase 1 (TAK1) plus its associated proteins TAB1 and TAB2 (Takaesu, Kishida et al. 2000; Jiang, Ninomiya-Tsuji et al. 2002) and TAB3 (Cheung, Nebreda et al. 2004) (see **Figure 1.2**).

1.7.4 TAK1

TAK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) that is directly linked to the activation of NF-κB, which is the transcription factor responsible for the upregulation of pro-inflammatory cytokines. As the activated TRAF6: IRAK-1 heterodimer binds with membrane-bound TAK1: TAB1: TAB2: TAB3 complex, an as yet unknown kinase phosphorylates TAK1 and TAB2 thus releasing all components, excluding IRAK-1, into the cytosol (Jiang, Ninomiya-Tsuji et al. 2002). TAB2 and TAB3 contain conserved zinc finger

domains that bind TRAF6 and act as adaptor molecules that allow TRAF6 to activate the kinase domain of TAK1 (Kanayama, Seth et al. 2004), where TAB1 acts to enhance TAK1 activation (Akira 2003), which leads to the release of NF-κB from inhibitory proteins (see **Figure 1.2**).

1.7.5 Canonical NF-κB activation

There are five proteins in the NF-κB (nuclear factor kappa enhancer binding protein) family of transcription factors, RelA (p65), RelB, c-Rel, p105 (NF-κB1) and p100 (NF-κB2). The most common NF-κB is made up of a dimer made up of p50 and RelA and is a member of the Rel family of transcription factors, which contain a conserved N-terminal Rel-homology domain (RHD) that encompasses the DNA-binding motif, dimerisation domains and the nuclear localisation sequence (NLS) (Mercurio and Manning 1999). In a resting cell, NF-κB is held in an inactive conformation by a complex of inhibitory proteins, IκBs. Six mammalian IκBs have been isolated that all possess ankyrin-like repeats, but NF-κB activity appears to be mainly controlled by three main proteins, IκBα, IκBβ and IκBε (Baeuerle and Baltimore 1996; Whiteside, Epinat et al. 1997). NF-κB is sequestered in the cytoplasm by IκBs, which bind to the carboxy terminus of the RHD and masks the NLS thus blocking its interaction with nuclear import machinery (Henkel, Zabel et al. 1992).

IκB kinases (IKKs) were identified as a large (700-900 kDa) cytoplasmic complex that contains two serine kinases, IKKα (IKK1) and IKKβ (IKK2) and a regulatory component IKKγ (NEMO). IKKα and IKKβ possess N-terminal kinase domains and two C-terminal protein: protein interaction motifs, a leucine-zipper and a helix-loop-helix motif (Mercurio, Zhu et al. 1997). Activated TAK1 targets the IKK complex and phosphorylates serine residues in the activation loop of IKKα and IKKβ, which leads to the serine phosphorylation of IκBα (ser32 and 36) and IκBβ (ser19 and 23) that targets these molecules for degradation by the 26S proteasome (Chen, Hagler et al. 1995). *In vitro* and *ex vivo* studies have revealed an essential role for IKKβ serine phosphorylation, but not for IKKα, suggesting IKKβ may play a more important role in NF-κB activation (Mercurio, Zhu et al. 1997). Once IκBs have been degraded and the NLS uncovered, NF-κB translocates to the nucleus to upregulate target genes (see **Figure 1.2**).

1.7.6 MAPK activation

NF-κB is the primary transcription factor that is activated upon the activation of the TLR/IL-1R signalling pathway; however other targets include the mitogen-activated protein kinases (MAPKs), which are involved in many fundamental cellular processes including the cell

growth, differentiation, stress and the inflammatory response. MAPKs are a group of serine/threonine protein kinases that are activated by a plethora of extracellular stimuli through the dual phosphorylation of conserved threonine and tyrosine residues (Cobb and Goldsmith 1995). There are three subcategories of MAPK, the extracellular signal regulated kinase (ERK), Jun N-terminal kinase (JNK), also known as the stress-activated protein kinase (SAPKs) and p38 MAPK.

The ERKs were the first of the MAPK family to be cloned (Boulton, Yancopoulos et al. 1990; Boulton, Nye et al. 1991) and it was found that phosphorylation of these molecules led to the activation of transcription factors such as p65TCF/Elk-1, c-jun and c-myc that are linked to cell growth and proliferation (Gille, Sharrocks et al. 1992; Davis 1993; Marais, Wynne et al. 1993).

The p38 MAPKs were originally identified as 32 kDa proteins that underwent tyrosine phosphorylation in response to endotoxin treatment and osmotic shock (Han, Lee et al. 1994). The original form of p38 is known as p38α and has been shown to be activated in response to IL-1 stimulation (Freshney, Rawlinson et al. 1994), however three other isoforms have been discovered, p38β (Jiang, Chen et al. 1996), p38γ (Lechner, Zahalka et al. 1996; Mertens, Craxton et al. 1996) and p38δ (Goedert, Cuenda et al. 1997; Jiang, Gram et al. 1997).

The p38 MAPKs are involved in the activation of the transcription factor activator protein-1 (AP-1), which is a heterodimer comprised of basic leucine zipper (bZIP) transcription factors, typically c-Jun and JunD, along with members of the *fos* (usually c-Fos) and ATF (usually ATF2) families. All bZIP transcription factors contain leucine zippers that enable homo- and heterodimerisation, and AP-1 components are organised into Jun-Jun, Jun-Fos, or Jun-ATF dimers. The p38 MAPKs can both lead to the direct activation of AP-1 by phosphorylating the trans-activating domain of AFT2 and lead to the upregulation of transcription of AP-1 components (Karin, Liu et al. 1997; Kato, Kravchenko et al. 1997). Activation of AP-1 allows translocation to the nucleus where it is involved in transcribing many genes related to inflammation including IL-1, TNF, c-Jun itself and various proteases and cell adhesion molecules such as E selectin (Karin, Liu et al. 1997; Read, Whitley et al. 1997).

JNKs are a family of proteins that are encoded by three genes, JNK1, JNK2 and JNK3 which form 10 different isoforms. These proteins are phosphorylated under many different

cellular stresses including inflammation. Like p38 MAPKs, JNKs are involved in the activation of the transcription factor AP-1.

The MAPKs are activated by a three-tiered phosphorylation cascade where the MAP kinase kinase kinase (MAPKKK) phosphorylates the MAP kinase kinases (MKKs) that activate the MAPKs. MKK3, MKK4 and MKK6 are the MKKs that are involved in the phosphorylation of p38 (Lin, Minden et al. 1995; Han, Lee et al. 1996; Moriguchi, Toyoshima et al. 1996). TAK1, a key mediator in NF-κB activation (**section 1.7.4**), is also a member of the MAPKKK family and is responsible for the bifurcation of the signalling pathways.

1.7.7 Summary of the TLR/IL-1 signalling pathway

Binding of ligand to TLR/IL-1R results in receptor clustering and recruitment of the adaptor molecule MyD88 to the receptor complex. MyD88 interacts with the receptor via its C-terminal Toll-interleukin 1 receptor (TIR) domain and links it to IL-1R-associated kinase (IRAK)-4 through the N-terminal death domain (DD) (Akira and Sato 2003). Activated IRAK-4 recruits and causes hyperphosphorylation of IRAK-1, which induces the interaction of tumour necrosis factor (TNF) associated factor (TRAF6). The formation of the Receptor: MyD88: IRAK1/4: TRAF6 complex (complex I) causes a conformational change that leads to the release of IRAK1:TRAF6 so it can interact with a preformed membrane-associated signalling complex that consists of the MAP 3-kinase TAK1 (Yamaguchi, Shirakabe et al. 1995) and three TAK1 binding proteins, TAB1, TAB2 and TAB3 (Shibuya, Yamaguchi et al. 1996) (complex II). TAB2 and TAB3 are adaptor proteins that facilitate the binding of TRAF6 to TAK1 (Takaesu, Kishida et al. 2000) and TAB1 activates its kinase activity (Jiang, Johnson et al. 2003). Complex III is formed as IRAK1 releases TRAF6: TAK1: TAB1: TAB2: TAB3 from the membrane to act on their cytosolic targets, whilst IRAK1 remains at the membrane where it is targeted for degradation (Jiang, Ninomiya-Tsuji et al. 2002). TAK1 binds and activates the inhibitory κB (IκB) kinase (IKK) complex that phosphorylates the NF-κB binding proteins IκBα/β and targets them for degradation (Muzio, Polentarutti et al. 2000). Once the NF-κB dimer is released it can translocate to the nucleus to act as a transcription factor for proinflammatory genes. An overview of the NF-κB signalling pathway can be found in **Figure 1.2.** Activation of TAK1 also leads to the activation of the MAPKKs MKK3, MKK4 and MKK6, which leads to the activation of p38 MAPK and thus the transcription factor AP-1.

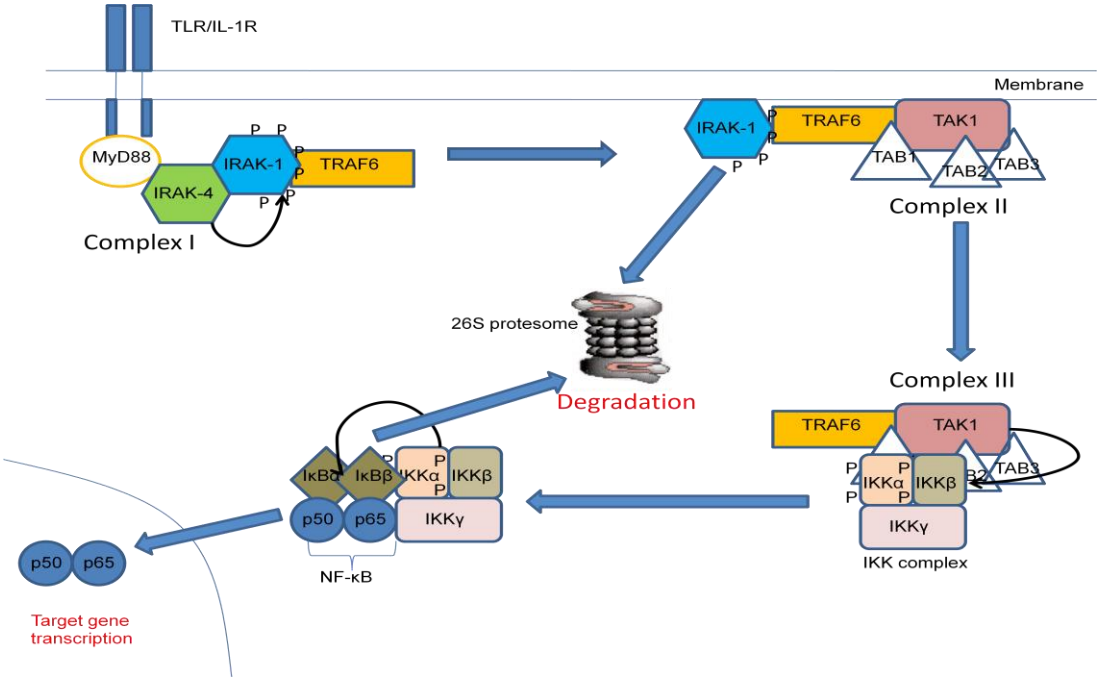


Figure 1.2: Overview of canonical NF-κB signalling

Upon activation of TLR/IL-1R at the membrane, complex I is formed as MyD88 is recruited to receptor complex, which leads to the hyperphosphorylation (P) of IRAK-1 by IRAK-4 and the subsequent binding of TRAF6. IRAK-1: TRAF6 heterodimer leaves the receptor and binds TAK1 and its binding proteins, TAB1/2/3 (complex II). Complex III is formed as IRAK-1 releases TRAF6: TAK1: TAB1/2/3 from the membrane, where it targets and activates the IKK complex by phosphorylation. P-IKK activates the transcription factor NF-κB by targeting its bound inhibitory proteins (IκBs) for degradation, thus allowing NF-κB to translocate to the nucleus to upregulate target genes.

1.8 The MyD88-independent pathways

All TLRs and IL-1R signal through the adaptor MyD88; with the exception of TLR3 (**section 1.10.1**) which signals through the TIR domain-containing adaptor inducing IFN-β (TRIF) and TLR4 which signals through both MyD88 and TRIF (also known as TICAM1). TRIF contains a TIR domain for TIR-TIR interactions and a C-terminal RIP homotypic interaction motif (RHIM), which mediates interaction with members of the receptor interacting protein (RIP) family (**Section 1.10.1.**) (Meylan, Burns et al. 2004).

MyD88 deficient mice are capable of activating NF-κB and MAPKs in a late phase response that is associated with the activation of Type I interferons (IFNs) in response to LPS and viral stimulus (Kawai, Adachi et al. 1999; Kawai, Takeuchi et al. 2001), which led to the discovery of TRIF. TRIF deficient mice show early phase NF-κB and MAPK signalling, however these mice show a reduction in proinflammatory cytokine and Type I IFN production (Hoebe, Du et al. 2003; Yamamoto, Sato et al. 2003). These data suggest that TRIF is required for Type I IFN induction and maximal cytokine production in response to LPS. TRIF has been shown not to bind directly to TLR4 but is linked by another adaptor molecule TRIF-related adaptor molecule (TRAM), which is required for TRIF-dependent signalling in response to LPS (Yamamoto, Sato et al. 2003).

TLR3 binds to double-stranded RNA that is produced by many viruses during replication (Alexopoulou, Holt et al. 2001) and poly(I:C) is a synthetic double-stranded RNA and TLR3 agonist. MyD88$^{-/-}$ and TRAM$^{-/-}$ mice respond normally to poly(I:C), however TRIF$^{-/-}$ mice do not induce Type I IFNs or activate NF-κB or MAPKs in response to poly(I:C) implying that TRIF is the sole adaptor for TLR3 signalling (Yamamoto, Sato et al. 2003) (**see section 1.10.1**). An overview of MyD88-independent signalling can be found in **Figure 1.3**.

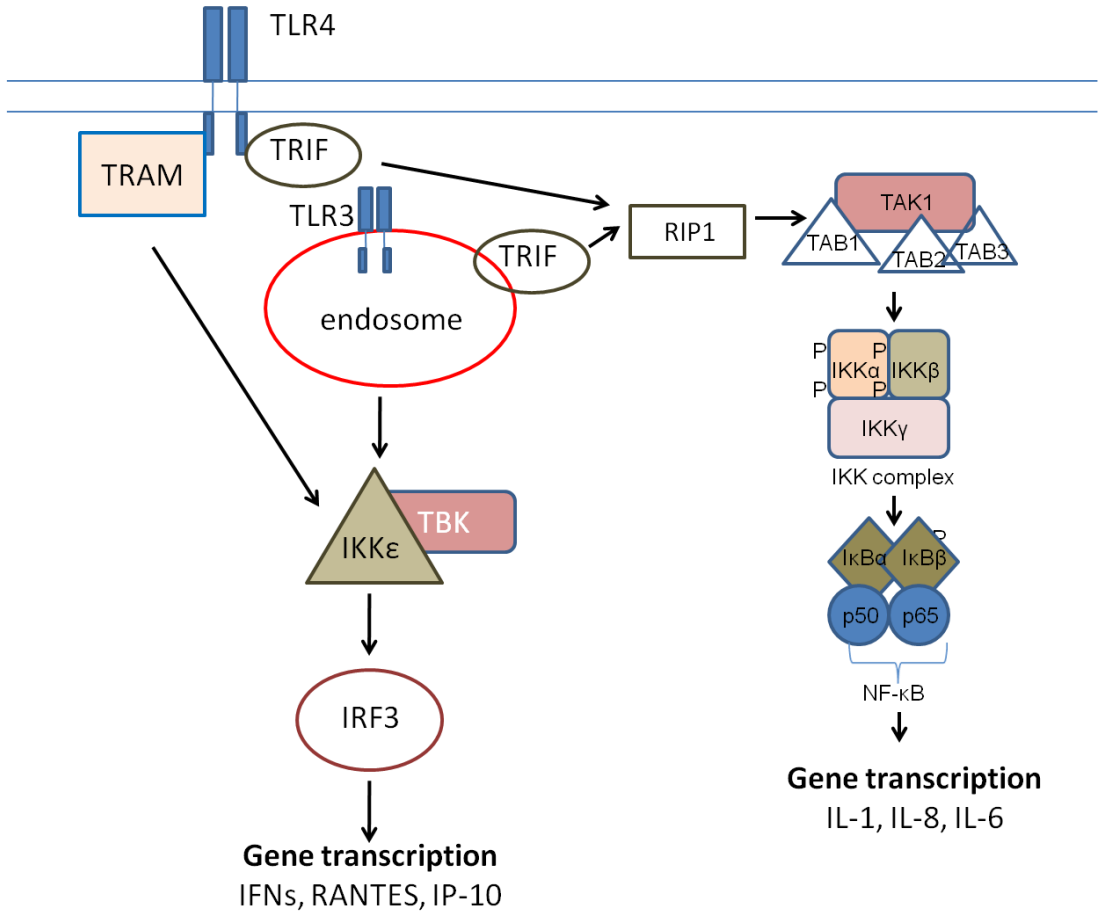


Figure 1.3: An overview of MyD88-independent signalling

TLR3 is activated in response to viral dsRNA, which leads to the activation of the adaptor molecule TRIF. TRIF recruits RIP1, which leads to the activation of the NF-κB transcription factor through TAK1 and the IKK complex. Activated NF-κB leads to the upregulation of target genes including IL-1, IL-6 and IL-8. Activated TLR3 can also activate the IRF3 transcription factor through the IKKε/TBK kinases, which leads to the upregulation of the IFNs, RANTES and IP-10. TLR can also activate MyD88-independent pathways through TRIF, leading to NF-κB, and TRAM leading to IRF activation.

1.9 The non-canonical NF-κB signalling pathway

Two members of the NF-κB family of transcription factors, p100 and p105, are produced as precursor proteins that are processed by proteolytic cleavage into active transcription factors, p52 and p50, respectively. These proteins are involved in the activation of non-canonical NF-κB signalling pathways. The precursor proteins serve the same function as the IκBs in canonical NF-κB signalling in that they keep the transcription factor in the inactive form in the cytoplasm. Upon activation of the p100/p105 signalling pathways, the precursor proteins are partially degraded to remove their C-terminal domains that include IκB-like ankyrin repeats, which release the active p52/p50 fragments. Despite p52 and p50 containing the RHD that allows them to bind to DNA, they lack the transactivation domain (TAD) that is required to activate gene transcription, so these active fragments require the binding of RelB, c-rel or p65 to form heterodimers to activate transcription (Hayden and Ghosh 2004). Homodimers of p50 or p52 have even been shown to inhibit gene transcription (Zhong, May et al. 2002).

The transcription factor p105 undergoes constitutive processing to p50 fragments by partial degradation by the proteasome. This is a co-translational mechanism that is thought to be involved in the generation of p50 fragments for p50/p65 canonical NF-κB heterodimers (Fan and Maniatis 1991; Palombella, Rando et al. 1994; Lin, DeMartino et al. 1998). Partial processing of p105 is due to a glycine-rich region (GRR) located between residues 376 and 404 of p105 that halts proteasomal degradation (Lin and Ghosh 1996; Orian, Schwartz et al. 1999). There is evidence to suggest that p105 can undergo induced processing via phosphorylation of C-terminal serines by IKKβ in response to proinflammatory stimuli such as LPS, IL-1 and TNFα, however the functional role of this degradation is yet to be elucidated (Fujimoto, Yasuda et al. 1995; MacKichan, Logeat et al. 1996; Heissmeyer, Krappmann et al. 1999; Orian, Gonen et al. 2000; Lang, Janzen et al. 2003). Constitutive p105 processing is inhibited when it forms homo- or heterodimeric complexes with other NF-κB proteins (Harhaj, Maggirwar et al. 1996; Cohen, Orian et al. 2001), which suggests its role is dependent of the availability of NF-κB proteins in the cellular milieu.

Unlike p105, processing of p100 is a tightly controlled mechanism with only minimal p100 processing in unstimulated cells (Heusch, Lin et al. 1999). The main function of p100 is to keep the transcription factor RelB in the cytoplasm and to regulate its transcriptional activity (Dobrzanski, Ryseck et al. 1995; Solan, Miyoshi et al. 2002). The processing of p100

has been linked to the activation of receptors involved with the immune response including LTβR (Dejardin, Droin et al. 2002), BAFF-R (Kayagaki, Yan et al. 2002) and CD40 (Coope, Atkinson et al. 2002). LTβR binds to members of the TNF superfamily and activation of BAFF-R and CD40 is linked to B-cell maturation and survival. The bacterial PAMP LPS and the cytokine TNF-like weak inducer of apoptosis (TWEAK) have also been shown to activate the p100 processing (Mordmuller, Krappmann et al. 2003; Saitoh, Nakayama et al. 2003). Despite other TNF family members activating this pathway, several studies have shown that TNFα does not induce p100 processing (Coope, Atkinson et al. 2002; Dejardin, Droin et al. 2002; Derudder, Dejardin et al. 2003), but does lead to the upregulation of the canonical NF-κB targets, p100 and RelB (Derudder, Dejardin et al. 2003). It is important to note that the kinetics of p100 processing in the non-canonical NF-κB signalling pathway occurs over several hours, which is much slower than that of canonical NF-κB signalling that occurs in minutes (Beinke and Ley 2004).

Activation of the p100 non-canonical pathway has been linked to the NF-κB binding kinase (NIK). Overexpression of NIK leads to p100 processing to p52 (Senftleben, Cao et al. 2001) and splenocytes from mice that have a mutation in the NIK gene, the alymphoplasia mouse, have significantly reduced levels of p52 despite normal levels of p100 (Yamada, Mitani et al. 2000; Xiao, Harhaj et al. 2001). The kinase domain of NIK shares sequence homology with that of the MAP3Ks and has been shown to interact with (Regnier, Song et al. 1997) and phosphorylate IKKα (Ling, Cao et al. 1998). However, in contrast to canonical NF-κB signalling, binding of IKKα by NIK is independent of IKKβ and NEMO (Senftleben, Cao et al. 2001; Xiao, Harhaj et al. 2001). IKKα has been shown to directly phosphorylate p100, leading to its processing to p52 (Senftleben, Cao et al. 2001).

The non-canonical NF-κB signalling pathways have not been as extensively studied as canonical NF-κB signalling and it is likely that other mediators and activators of this pathway are, as yet, unidentified. The key mediators involved in non-canonical NF-κB signalling are outlined in **Figure 1.4**.

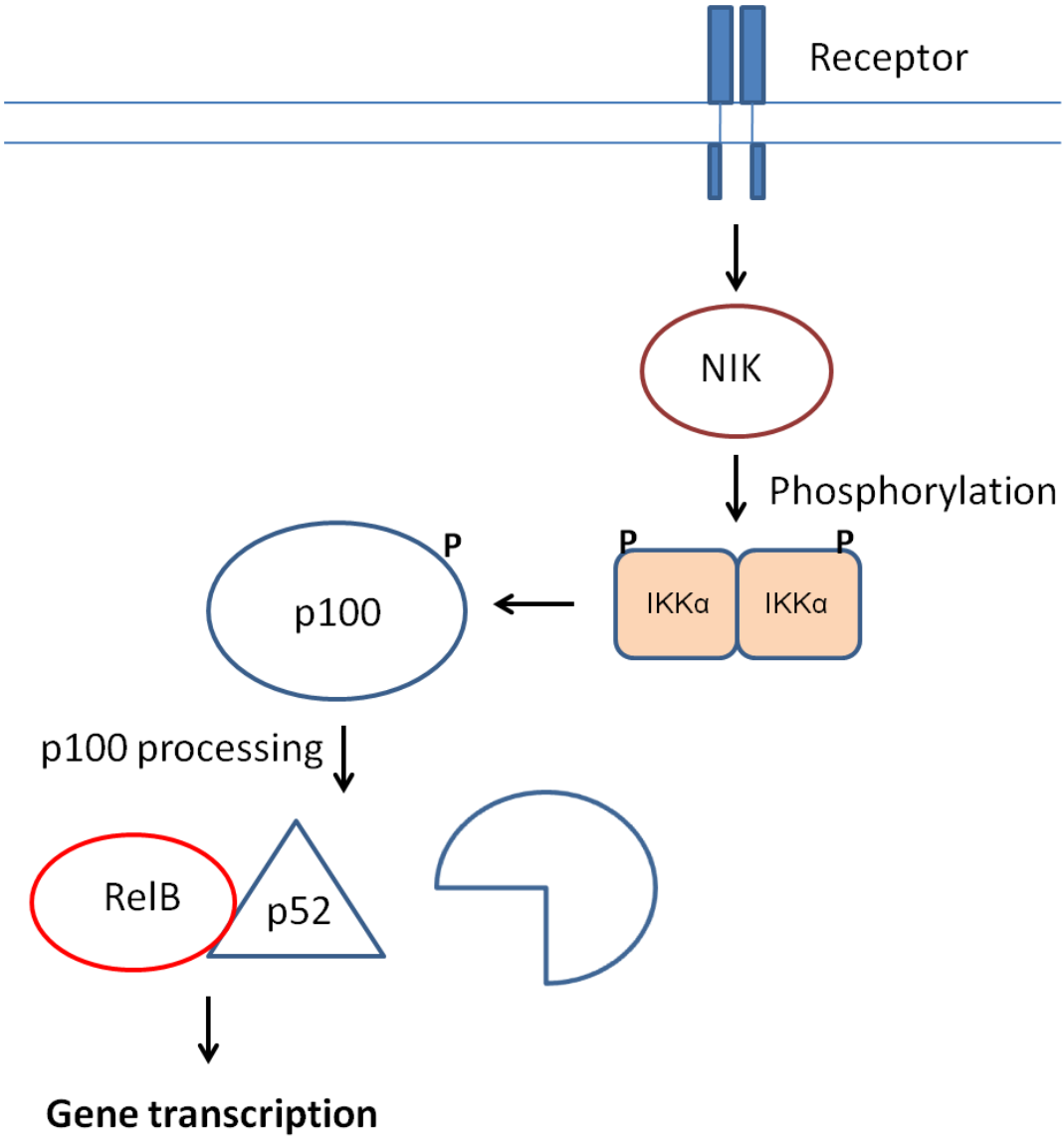


Figure 1.4: The key mediators of the non-canonical NF-κB signalling pathway

Activation of the non-canonical signalling pathway is mediated through the kinase NIK that is involved in phosphorylating an IKK complex comprised of two IKKα subunits. The activated IKK complex phosphorylates p100, which leads to its processing into the active p52 fragment. P52 dimerises with RelB, which contains the transactivation domain (TAD), allowing for translocation to the nucleus and subsequent upregulation of target genes.

1.10 Detection of virus

Viruses cause some of the most frequent infections in humans. Since viral replication occurs in the cytoplasm, the host defence systems that recognise virus are either located within the cytoplasmic compartment or in endosomes. The main receptors involved in detecting virus are TLR3 and RIG-I/MDA5.

1.10.1 TLR3 signalling

Toll-like receptor 3 (TLR3) was shown to bind to double-stranded RNA (dsRNA) (Alexopoulou, Holt et al. 2001), which is either produced during viral replication or is part of the viral genome (Jacobs and Langland 1996). Originally TLR3 was shown not to recognise single-stranded RNA (ssRNA) (Alexopoulou, Holt et al. 2001), however more recent data suggests that it is capable of binding to ssRNA (Marshall-Clarke, Downes et al. 2007). TLR3 also binds to the stable synthetic dsRNA, poly(I:C) and TLR3 binds to this agonist preferentially over virus-derived dsRNA (Okahira, Nishikawa et al. 2005). Activation of TLR3 leads to the induction of type I IFN and activation of NF-κB by distinct pathways.

TLR3 has a similar structure to that of other TLRs, being a type I transmembrane receptor (Jin and Lee 2008). TLR3 is a monomer is solution but can form homo-multimers within the membrane, which occurs upon ligand binding (Bell, Botos et al. 2006). TLR3 is mainly expressed on endosomes, however many cell types, including epithelial cells, have also been shown to express TLR3 on the cell surface (Cario and Podolsky 2000; Heinz, Haehnel et al. 2003) but the functional role of surface TLR3 is unclear. TLR3 binds to dsRNA in the endosome and this binding is dependent on acidic pH (de Bouteiller, Merck et al. 2005).

Activation of TLR3 leads to its phosphorylation at two tyrosine residues in its C terminus (Sarkar, Peters et al. 2004) and this leads to the recruitment of the adaptor molecule TRIF (**see section 1.8**). Stimulation of this pathway leads to the activation of distinct pathways leading to the activation of the IRFs and NF-κB transcription factors. Activation of the IRF signalling pathway and subsequent induction of the type I and type III IFNs (**see section 1.11.4**) occurs through TRIF binding to TRAF3, which is responsible for recruiting the kinases involved in phosphorylating and activating the IRFs, TANK binding kinase 1 (TBK1) and IKKε (Hacker, Redecke et al. 2006; Oganesyan, Saha et al. 2006).

NF-κB is also activated by TLR3 through the receptor interacting protein 1 (RIP1) protein. TRIF contains a C-terminal RIP homotypic interaction motif that mediates its binding to proteins of the RIP family and activation of this signalling pathway leads to the recruitment

of RIP1 to the TLR3-TRIF signalling complex (Meylan, Burns et al. 2004). Another member of the RIP family of proteins, RIP3, has shown to be a negative regulator of TLR3-mediated NF-κB activation (Meylan, Burns et al. 2004). Recruitment of RIP1 leads to the activation of TAK1 and subsequently the IKK complex leading to IκB degradation, as is seen in canonical NF-κB signalling (Cusson-Hermance, Khurana et al. 2005). It is likely that there are other mediators acting between RIP1 and TAK1, however these are yet to be identified but TRAF6 has been suggested as a possible mediator as TRIF contains 3 TRAF6-binding motifs and over expression of dominant negative TRAF6 leads to inhibition of TRIF-induced NF-κB activation (Sato, Sugiyama et al. 2003; Jiang, Mak et al. 2004).

1.10.1.1 IRFs

Interferon regulatory factors (IRFs) are a family of 9 different transcription factors (IRF1-9) involved in the induction of the Type I and III IFNs. All IRF proteins contain an N-terminal DNA binding domain consisting of 5 conserved tryptophan-rich repeats, which forms a helix-turn-helix that recognises the interferon regulatory element upstream of IRF-regulated genes (Mamane, Heylbroeck et al. 1999; Taniguchi, Ogasawara et al. 2001). The C-terminal domains of IRFs are less conserved and mediate interactions with other transcription factors and signalling mediators. Despite the disparity in the C-terminal, all of the IRFs, except IRF1 and IRF2, contain the Mad-homology 2 (MH2) domain that are found in the Smad family of transcription factors (Fujii, Shimizu et al. 1999).

IRF3 and IRF7 have been found to be the key IRFs involved in antiviral immunity and have found to be involved in activating the transcription of type I and III IFNs in response to virus (Honda and Taniguchi 2006). Despite IRF3 and IRF7 being the prototypic IRFs in antiviral signalling, IRF1 and IRF5 have also been shown to play a role in antiviral immunity (Mamane, Heylbroeck et al. 1999; Taniguchi, Ogasawara et al. 2001), although do not seem to play an essential role in type I IFN induction in response to virus (Takaoka, Yanai et al. 2005). IRF3 is constitutively expressed and resides in the inactive form in the cytosol; upon viral infection, it is phosphorylated at specific serines in the C-terminal regulatory domain (Honda and Taniguchi 2006). Phosphorylation of IRF3 leads to either its homodimerisation or heterodimerisation with IRF7 and assembly of the nuclear holocomplex. The holocomplex is made up of IRF dimers associated with co-factors, either cyclic AMP responsive element binding protein (CREB) binding protein (CBP) or p300, which together are involved in binding to DNA and upregulating the transcription of target genes (Lin, Heylbroeck et al. 1998; Sato, Tanaka et al. 1998; Weaver, Kumar et al. 1998; Yoneyama, Suhara et al. 1998).

In contrast to IRF3, IRF7 is only expressed in small amounts in most cell types, but is strongly induced by type I IFN-mediated signalling. Binding of type I IFNs to IFN receptor leads to the upregulation of IRF7, suggesting it is involved in late-phase IFN production working in a positive feedback loop. IRF7 is activated in a similar way to IRF3 by phosphorylation of serines in the C-terminal domain, which leads to homo- or heterodimerisation with IRF3 and translocation to the nucleus. The different combinations of IRF3/7 homo-/heterodimers have differential effects on gene transcription as IRF3 is a potent activator of *IFNB* genes, but not of *IFNA* (except for *IFNA4*) and IRF7 activates both *IFNB* and *IFNA* genes (Marie, Durbin et al. 1998; Sato, Hata et al. 1998; Sato, Suemori et al. 2000).

It was originally thought that only IFR3 was involved in the early phase induction of *IFNB* genes and this production of type I IFNs leads to the production of IRF7 for the late phase induction of *IFNA* and *IFNB* genes. However, irf7$^{-/-}$ MEFs showed a severe impairment in type I IFN induction in response to several ssRNA viruses, suggesting that the small amount of IRF7 constitutively produced is crucial for early phase IFN production (Honda, Yanai et al. 2005).

1.10.2 RIG-I/MDA5 signalling

TLR3 was thought to be the key mediator in viral detection within the host cells until a study discovered that TLR3 knockout cells produced IFN in response to both poly(I:C) stimulation and viral infection (Yoneyama, Kikuchi et al. 2004). In 2004 Retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which are part of the RIG-I-like receptor family of proteins, were found to be involved in sensing viral RNA in the cytoplasm (Yoneyama, Kikuchi et al. 2004).

RIG-I and MDA5 both contain two repeated N-terminal caspase recruitment domains (CARDs) and a C-terminal DExD/H-box RNA helicase domain and these structures show sequence similarity (23% and 35%, respectively). The CARD domains have been shown to be involved in the activation of IRF3, IRF7 and NF-κB and the subsequent production of IFN-β (Yoneyama, Kikuchi et al. 2004). The RNA helicase domain has been shown to bind directly to poly(I:C) in an ATP-dependent manner (Saito, Hirai et al. 2007). The C-terminus of RIG-I has been shown to act as a repressor domain (RD) that keeps it in the latent form in the cytosol (Saito, Hirai et al. 2007).

Cardif (also known as MAVS/VISA/IPS-1) was identified as a CARD-containing adaptor molecule of RIG-I/MDA5 signalling and analysis of Cardif knockout cells suggests its

involvement in antiviral signalling (Kumar, Kawai et al. 2006; Sun, Sun et al. 2006). Cardif is localised to the outer membrane of the mitochondria (Li, Sun et al. 2005; Lin, Lacoste et al. 2006) and this localisation is essential for its role in activating TBK/IKKε and RIP1/IKKα/IKKβ kinase complexes that are involved in activating IRF3 and NF-κB, respectively. Lysine-63 polyubiquitination of RIG-I, but not MDA, has been shown to be essential for RIG-I/MDA5 signalling and the E3 ubiquitin ligase TRIM25 has been shown to be involved in this process (Gack, Shin et al. 2007). TRAF3 has also been shown to be involved in RIG-I signalling as it binds to Cardif through a TRAF-interacting domain (TIM) and is involved with type I IFN release (Saha, Pietras et al. 2006).

RIG-I and MDA5 recognise different viruses as RIG-I knockout MEFs infected with Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), Sendai virus (SeV), influenza virus or Japanese encephalitis virus (JEM) or stimulated with *in vitro* transcribed dsRNA exhibited impaired IFN release, whereas IFN release was unaffected by these viruses in MDA5 deficient MEFs . However MDA5 deficient MEFs showed impaired IFN release in response to infection with viruses belonging to the Picornaviridae family of viruses, which is not seen in RIG-I deficient MEFs (Loo, Fornek et al. 2008). Recently it has been discovered that RIG-I binds to a 5'-triphosphate moiety in viral RNA that is usually capped in host endogenous mRNA (Hornung, Ellegast et al. 2006; Pichlmair, Schulz et al. 2006), however the structure that MDA5 recognises is yet to be elucidated.

1.11 Endpoint of signalling: Cytokine production

Cytokines are proteins (8-30 kDa in size) that can act over a range of distances (auto-, para-, and endocrine) on a variety of different cells types including leukocytes and epithelial cells. The main function of cytokines is to orchestrate the immune response by activating, inhibiting or drawing leukocytes to the site of infection by chemotaxis. In contrast to hormones, cytokines can be produced from a variety of different cell types and almost every nucleated cell. Epithelial, endothelial and resident macrophages in particular, are capable of producing IL-1, IL-6 and TNFα (Cannon 2000).

Upon stimulation of the TLR/IL-1R signalling pathway in response to infection, activation of NF-κB and other transcription factors lead to the production of cytokines; some of which are pro- and others that are anti-inflammatory. Locally, proinflammatory cytokines stimulate leukocyte proliferation, cytotoxicity, release of proteolytic enzymes, and synthesis of prostaglandins and initiate a cascade of "secondary" anti-inflammatory cytokine synthesis and secretion (Cannon 2000). Systemically, proinflammatory cytokines

raise the thermoregulatory set point to induce fever and caused sequestration of iron into intracellular sites to create a hostile environment for the growth of certain bacteria. Cytokines act on the central nervous system to reduce locomotive activity and increase slow wave sleep to reduce energy consumption. Skeletal muscle is broken down to provide amino acids for acute phase plasma proteins. Acute phase proteins are synthesised by the liver and work with leukocytes to clear pathogens. Acute phase proteins include complement factors, C-reactive protein and serum amyloid P, which are involved in opsonisation of dead cells, chemotaxis and target cell lysis (Petersen, Nielsen et al. 2004).

There are over 80 different cytokines that have been discovered to date. The cytokines that are used as a pro-inflammatory stimulus or a measure of the inflammatory response throughout this thesis are discussed individually.

1.11.1 Interleukin 1

IL-1 is a NF-κB-regulated cytokine that is produced by cells in response to pathogens (**Figure 1.5**). There are two forms of agonistic IL-1, IL-1α and IL-1β. They are independent gene products that are found in the same 430-kb gene cluster on human chromosome 2q13 (Nicklin, Weith et al. 1994). Both IL-1α and IL-1β are pro-inflammatory cytokines that bind to the same receptors, but IL-1α is mainly trapped in the cytosol and is only seen systemically during severe disease. IL-1β is the common mediator of systemic inflammation.

IL-1β is produced as an inactive proform that is cleaved by caspase 1 to produce active IL-1β. IL-1β is produced largely by immune cells including monocytes, macrophages, dendritic cells and B- and T- cells but can also be produced by epithelial and endothelial cells (Cannon 2000). IL-1β is a potent pro-inflammatory cytokine that is capable of detrimental effects on host tissues. As such, a network of regulatory mechanisms has evolved that attenuates IL-1 potency. IL-1 is regulated by an endogenous antagonist, IL-1ra. IL-1ra is a 17-kDa (152 amino acids) protein that binds with high affinity to the IL-1RI and a somewhat lower affinity to IL-1RII (Dripps, Verderber et al. 1991). IL-1ra share similar amino acid sequences, gene structure and chromosomal location (2q13) (Nicklin, Weith et al. 1994) with that of IL-1α and IL-1β (Arend 1993). The crystal structure of IL-1ra bound to IL-1RI has been resolved to 2.7 Å (Schreuder, Tardif et al. 1997) and this showed that IL-1ra and IL-1β have an indistinguishable β-pleated sheet structure and both bind in the cleft formed between the N-terminal immunoglobulin (Ig) domains. Unlike IL-1β, IL-1ra is unable to

contact the third Ig domain and therefore cannot initiate downstream signalling (Greenfeder, Varnell et al. 1995), revealing IL-1ra to be a competitive inhibitor of IL-1.

1.11.2 Interleukin 6

Interleukin 6 (IL-6) belongs to the 4 α-helix haematopoietic family of cytokines and is 212 amino acids in length. The IL-6 gene has been mapped to human chromosome 7p21 and contains 4 introns and 5 exons. IL-6 molecular weight can vary between 22- and 28-kDa, depending on the phosphorylation and glycosylation state of the cytokine (Heinrich, Behrmann et al. 1998). Another classic NF-κB regulated gene, *IL*-6 is not constitutively expressed, but is upregulated in response to a variety of PAMPs and DAMPs including viral infection, LPS, IL-1β, TNFα and INF-γ (**Figure 1.5**). IL-6 is produced by a wide variety of immune and non-immune cells, especially in macrophages, dendritic cells, lymphocytes, endothelial, epithelial and fibroblasts (Jawa, Anillo et al. 2010), although virtually every cell is capable of producing IL-6 (May, Ndubuisi et al. 1995) including muscle cells (Petersen and Pedersen 2005).

IL-6 acts as both a pro- and anti-inflammatory cytokine and has a key role in mediating the immune and acute phase response, as demonstrated by the IL-6 knockout mouse (Kopf, Baumann et al. 1994). IL-6 has a variety of effects on the immune system, including having an important role in immune cell maturation where it is involved in the differentiation of T cells and induction of antibody production in B cells (Naka, Nishimoto et al. 2002), however does not seem to play a role in the proliferation of activated B cells (Park and Pillinger 2007). IL-6 targets endothelial cells and induces them to produce chemokines and adhesion molecules that attract leukocytes to the site of inflammation (Cronstein 2007). IL-6 induces the production of phospholipase A_2 (PLA_2), which in turn leads to the production of other key pro-inflammatory mediators including leukotrienes, prostaglandins and platelet-activating factor (PAF) (Tilg, Dinarello et al. 1997). PAF is involved in the priming of peripheral blood neutrophils and this action is potentiated by IL-6 (Biffl, Moore et al. 1994).

Inflammation is a tightly controlled process to avoid unnecessary damage to the host and among the pleiotropic effects of IL-6 are important anti-inflammatory functions. IL-6 has been shown to induce the production of IL-1ra and soluble TNFR to down regulate the effects of the potent pro-inflammatory cytokines IL-1 and TNFα, respectively, *in vivo* (Tilg, Dinarello et al. 1997). IL-6 also induces the tissue inhibitor of matrix metalloproteinases (TIMP) (Jawa, Anillo et al. 2010), which reduces cell damage.

IL-6 binds to a heterotrimeric receptor complex made up of an IL-6Rα subunit and two gp130 subunits (Bravo and Heath 2000). IL-6Rα is an 80 kDa protein that can be membrane bound or a soluble form and many pro-inflammatory stimuli cause IL-6Rα shedding including host-mediated responses such as neutrophil degranulation, bacterial products such as pore forming toxins and the synthetic chemoattractant f-met-leu-phe (Walev, Vollmer et al. 1996; Jawa, Anillo et al. 2010). Soluble IL-6Rα bound to IL-6 to acts a potent activator for gp130, which is expressed on most cell types, thus allowing IL-6 signalling to occur in cells that do not express the membrane bound form of IL-6Rα (Rose-John, Scheller et al. 2006). Upon activation of the IL-6 receptor, the JAK/STAT pathway is activated (primarily JAK1 and STAT5) and leading to transcription of IL-6-stimulated genes (Bravo and Heath 2000).

1.11.3 Chemokines

Chemokines are a subset of cytokines that act as chemoattractants for immune cells such as monocytes and neutrophils to recruit them to the site of infection or injury. Chemokines are small (8-12 kDa) proteins that are subgrouped by their primary structure, which correlates with functional parameters. There are four different groups of chemokines, CC, CXC, CX3C and C, which are defined by the position of two conserved cysteines in the N-terminal region of the chemokine domain (Murphy 1994). The two main groups of chemokine are CXC and CC, where CXC chemokines are mainly involved in the recruitment of neutrophils and the CC chemokines in the recruitment of monocytes, lymphocytes and basophils (Choi and An 2011). Chemokines have a conserved three-dimensional structure that consists of a flexible N-terminus followed by three-stranded anti-parallel sheet and a C-terminal α-helix (Proudfoot 2002). This structure is recognised by its cognate chemokine receptor, which are G-protein coupled receptors made up of 7 transmembrane helices and downstream signalling is mediated by the activation of the associated heterotrimeric G-proteins (Murphy 1994; Strader, Fong et al. 1994).

1.11.3.1 Interleukin 8

Interleukin-8 (IL-8 or CXCL8) a classic NF-κB target gene (**Figure 1.5**), is a chemokine that is a potent chemoattractant for neutrophils. IL-8 is produced by a variety of cell types including monocytes, neutrophils, T lymphocytes, fibroblasts, endothelial and epithelial cells. IL-8 is a CXC chemokine that attracts neutrophils but not monocytes (Schroder, Mrowietz et al. 1987; Walz, Peveri et al. 1987; Yoshimura, Matsushima et al. 1987). The predominant form of IL-8 is 72 amino acids long, however endothelial cells produce a 77 amino acid variant of IL-8 that is thought to be involved in neutrophil adherence, which is

required prior to transmigration (Huber, Kunkel et al. 1991). The 72 amino acid version of IL-8 has also been shown to be a T-lymphocyte chemoattractant (Larsen, Anderson et al. 1989), have angiogenic activity (Koch, Polverini et al. 1992), and stimulates histamine release from basophils (Dahinden, Kurimoto et al. 1989; White, Yoshimura et al. 1989).

There is a strong link between IL-8 and respiratory inflammatory diseases as there are increased IL-8 levels in the bronchoalveolar lavage (BAL) fluid of both asthma (Fahy, Kim et al. 1995) and COPD (Keatings, Collins et al. 1996) patients. This increase in IL-8 has been shown to be a key factor in the production of a neutrophilic inflammatory environment in the airways of these patients (Fahy, Kim et al. 1995).

IL-8 is capable of binding to two different receptors, CXCR1 and CXCR2, which are expressed on a variety of cell types including a variety of leukocytes and non-haematopoietic cells such as epithelial and endothelial cells. However, these receptors are greatly associated with cells of a myeloid lineage and particularly with the chemoattraction and activation of neutrophils (Stillie, Farooq et al. 2009). Signal transduction through CXCR1 and CXCR1 plays a role in the antimicrobial activity of neutrophils including chemotaxis, degranulation and oxidative burst (Baggiolini and Clark-Lewis 1992). CXCR1 has shown to play a more dominant role in neutrophil chemotaxis than CXCR2 (Hammond, Lapointe et al. 1995; Quan, Martin et al. 1996).

1.11.3.2 RANTES

An IRF-stimulated gene regulated upon activation in normal T-cells, expressed and secreted (RANTES) (**Figure 1.5**), also known as CCL5, is a CC-type of chemokine and was discovered in a T- versus B-leukocyte differential screen. RANTES is an 8 kDa protein that is encoded by the *ccl5* gene located on chromosome 17. RANTES production is inducible upon viral stimulus of epithelial cells and mitogen or antigen stimulus of T-cell lines and circulating lymphocytes (Schall, Bacon et al. 1990). RANTES is a potent monocyte chemoattractant, but has no effect on neutrophil chemotaxis and is less effective at causing monocyte exocytosis that other CC chemokines such a MCP-1 (Schall, Bacon et al. 1990; Uguccioni, D'Apuzzo et al. 1995). RANTES has been shown to be an important chemotactic agent for many subsets of T lymphocytes such as $CD4^+$ and $CD45R0^+$ in endothelial-free assays (Schall, Bacon et al. 1990) and $CD4^+$ and $CD8^+$ in transendothelial systems, where it is the most potent CC chemokine for $CD8^+$ T cells (Roth, Carr et al. 1995). RANTES can attract and activate natural killer (NK) cells (Taub, Sayers et al. 1995; Maghazachi, Al-Aoukaty et al. 1996) and also attract eosinophils, which can secrete it (Rot, Krieger et al. 1992; Lim, Wan et al. 1996). RANTES targets basophils and causes them to release histamine (Kuna, Reddigari et al. 1992). RANTES has been shown to bind to four different chemokine receptors, CCR1 (Gao, Kuhns et al. 1993; Neote, DiGregorio et al. 1993), CCR3 (Daugherty, Siciliano et al. 1996), CCR4 (Power, Meyer et al. 1995) and CCR5 (Combadiere, Ahuja et al. 1996; Raport, Gosling et al. 1996).

1.11.3.3 IP-10

Another IRF-stimulated gene interferon-γ-induced protein 10 (IP-10) (**Figure 1.5**), also known as CXCL10, is a CXC chemokine that is produced in response to viral stimulus. The *cxcl10* gene is located on human chromosome 4 in a gene cluster with other CXC chemokines and encodes an 8.7 kDa protein. IP-10 is produced in a variety of different cell types including monocytes, keratinocytes, fibroblasts, T lymphocytes, endothelial and epithelial cells in response to Interferon (IFN)-γ (Luster, Unkeless et al. 1985). Interferon-γ administration in the mouse leads to increased expression of IP-10 in liver, kidney and lower levels in spleen (Narumi, Wyner et al. 1992). IP-10 does not attract or activate neutrophils (Dewald, Moser et al. 1992) however it has been shown to be involved in the chemotaxis of T-lymphocytes (Murphy, Tian et al. 1996; Taub, Longo et al. 1996) and tumour-infiltrating leukocytes (Liao, Rabin et al. 1995). IP-10 has also been show to be anti-

angiogenic (Angiolillo, Sgadari et al. 1995). IP-10 binds the G-protein coupled receptor CXCR3 (Loetscher, Gerber et al. 1996).

1.11.4 Interferons

The interferons (IFNs) are family of IRF-stimulated cytokines (**Figure 1.5**) that share significant amino acid homology and exhibit similar cellular effects on target cells including antiviral immunity, antiproliferative effects and modulation of the immune response. There are three major subgroups of IFNs, Type I, Type II and Type III. Type I INFs include IFNα, IFNβ, IFNω, IFNκ and IFNε; the only Type II IFN is IFNγ; and the more recently discovered Type III IFNs include IFNλ1, IFNλ2 and IFNλ3.

Type I interferons have potent antiviral activity by upregulating proteins that bind to dsRNA (Williams 1999), inhibit protein translation (Kerr and Brown 1978; Jacobs and Langland 1996), inhibit trafficking of viral ribonucleoprotein complexes (Weber, Haller et al. 2000) and induce apoptosis in virally infected cells (Takizawa, Ohashi et al. 1996; Der, Yang et al. 1997; Balachandran, Kim et al. 1998; Yeung, Chang et al. 1999; Gil and Esteban 2000). The essential role of these IFNs in antiviral immunity has been clearly demonstrated by the Type I IFN receptor knockout mouse, which is highly susceptible to viral infections (Hwang, Hertzog et al. 1995; Steinhoff, Muller et al. 1995). The genes encoding all different types of Type I IFNs are clustered on human chromosome 9 and share significant sequence homology (Diaz, Pomykala et al. 1994; Stark, Kerr et al. 1998).

In contrast, the Type II IFN, IFNγ, shares little amino acid sequence homology with the Type I IFNs (Stark, Kerr et al. 1998) and studies on the INF-γ receptor knockout mouse (Dalton, Pitts-Meek et al. 1993; Huang, Hendriks et al. 1993; Lu, Ebensperger et al. 1998) and humans with mutations in the IFN-γ receptor (Dorman, Picard et al. 2004) show that its primary role is not involved in antiviral immunity but has been linked to cell-mediated immune responses to intracellular pathogens and anti-tumour immune responses (Donnelly and Kotenko 2010).

Type III IFNs, IFNλ1, IFNλ2 and IFNλ3, have more recently been discovered as IFNs involved in the antiviral responses (Kotenko, Gallagher et al. 2003; Sheppard, Kindsvogel et al. 2003). The genes encoding INFλs are clustered on human chromosome 19 and resulting proteins share approximately 5-18% sequence homology with Type I IFNs (Donnelly and Kotenko 2010). IFNλ2 and IFNλ3 have almost identical amino acid sequences and upstream and downstream flanking sequences that is likely due to a duplication event (Donnelly and Kotenko 2010). Type III IFNs have been shown to be induced in response to the same

stimuli as its Type I counterparts (Coccia, Severa et al. 2004; Ank, West et al. 2006), which include many viruses and TLR agonists. Consistent with the joint roles in antiviral immunity of Type I and Type III IFNs, they are often co-expressed in viral infected cells (Kotenko, Gallagher et al. 2003; Sheppard, Kindsvogel et al. 2003). Virtually any nucleated cell can produce INFλs, however they have been strongly associated with human primary epithelial cells in response to respiratory syncytial virus (RSV) infection (Spann, Tran et al. 2004).

Each type of IFN binds a distinct receptor. Type I IFNs bind the IFNR1α and IFNR2α heterodimeric receptor complex; Type II IFN binds a tetrameric receptor complex consisting of two IFNγR1 and two IFNγR2 domains; and Type III IFNs bind IFNλR1 and IL-10R2 heterodimeric receptor complex. Activation of these receptors activate JAK/STAT pathways that lead to the transcription of IFN stimulated genes (ISG) through the binding of STATs to IFN stimulated response elements (IRSE) (Type I and III) or IFN-γ associated sequence (GAS) (Donnelly and Kotenko 2010).

1.12 TNFα signalling

1.12.1 TNFα

Tumour necrosis factor α (TNFα) is part of the large gene superfamily of the TNF-related superfamily proteins and was first identified as a product of lymphocytes and macrophages that can cause lysis to certain cell types including tumour cells (Granger, Shacks et al. 1969; Carswell, Old et al. 1975). TNFα has now been shown to have important roles in the immune system and the TNF receptor (TNFR) knockout mouse exhibits an increased susceptibility to microbial infections and a suppressed inflammatory response upon bacterial endotoxin challenge (Acton, Dahlberg et al. 1996; Steinshamn, Bemelmans et al. 1996). TNFα plays an important role in activating immune cells including monocytes and platelets and enhancing the ability of macrophages and NK cells to kill bacterial, viral and parasitic pathogens (Fiers 1991). TNFα has also been associated with sleep regulation (Shoham, Davenne et al. 1987) and causing cell death by apoptosis and necrosis (Beyaert and Fiers 1994).

TNFα is proinflammatory cytokine that is upregulated in response to acute inflammation. TNFα is encoded by a single copy gene that is located on human chromosome 6 and contains 4 exons and 3 introns, however 80% of the mature protein sequence is located in exon 4 (Idriss and Naismith 2000). TNFα is translated as a precursor protein that is 157 amino acids in length and has a molecular weight of 25 kDa (Vilcek and Lee 1991; Spriggs,

Deutsch et al. 1992). This precursor peptide contains a 76 amino acid presequence with a hydrophobic region that anchors TNFα to the membrane (Vilcek and Lee 1991). Membrane bound TNFα is proteolytically cleaved by TNFα converting enzyme (TACE) to produce the 17 kDa soluble form, which associates with two other TNFα soluble proteins to form the active homotrimer that are capable of activating the TNFR. TNFα is produced by a wide variety of immune and non-immune cells including macrophages, $CD8^+$ and $CD4^+$ T lymphocytes, B lymphocytes, NK cells, neutrophils, endothelial cells and smooth muscle cells (Vilcek and Lee 1991).

1.12.2 TNF receptor

TNFα binds to two different receptors, TNFR1 and TNFR2. TNFR1 is a 55 kDa protein made up of 434 amino acids and TNFR2 is a 75 kDa protein consisting of 439 amino acids (Loetscher, Steinmetz et al. 1991); both receptors bind TNFα homotrimers with a high affinity with a K_D in the nanomolar range (0.1 and 0.5 nM, respectively) (Idriss and Naismith 2000). TNFR1 and TNFR2 are type I transmembrane glycoprotein receptors that have very little sequence homology in their intracellular domains, which suggests they are involved in activating separate signalling pathways. TNFR1 is ubiquitously expressed on cells including fibroblasts and epithelial cells, however TNFR2 is only found on cells of hematopoietic origins (Armitage 1994). Most biological effects of TNFα are executed through TNFR1, whereas TNFR2 has few specific functions including induction of thymocyte proliferation and a possible sink for TNFα to reduce inflammation-mediated damage to host (Peschon, Torrance et al. 1998). Activation of TNFR1 has been shown to lead to the activation of the transcription factor NF-κB.

1.12.3 TNFR1 signalling pathway

TNFα signalling via TNFR1 is essential for a successful host defence response to a variety of pathogens (Flynn, Goldstein et al. 1995; Marino, Dunn et al. 1997) and overproduction of TNFα or the accumulation of TNFR1 in the membrane can have destructive consequences for the host as it has been associated with the pathogenesis of human diseases such as rheumatoid arthritis and multiple sclerosis (Korner, Lemckert et al. 1997; Raine, Bonetti et al. 1998; McDermott, Aksentijevich et al. 1999).

Upon activation of the TNFR1 by TNFα, the adaptor molecule TNF receptor-associated protein with a death domain (TRADD) and the serine-threonine kinase receptor interacting protein (RIP1) are recruited to the receptor complex (Chan 2007). TRADD and RIP1 bind to both TNFR1 and each other through C-terminal death domains (O'Donnell and Ting 2011).

Upon assembly of the activated receptor complex, TRADD recruits TRAF2 via its N-terminal TRAF binding domain. TRAF2 contains an N-terminal RING domain, followed 5 zinc finger domains and a C-terminal TRAF domain, which mediates homotrimerisation and binding with TRADD (Wajant, Pfizenmaier et al. 2003).

The role of RIP1 in TNFR1 signalling remains controversial as some groups have found that mouse embryonic fibroblasts from $RIP1^{-/-}$ mice have normal NF-κB activation (Wong, Gentle et al.), whereas others found RIP1 to be essential for NF-κB activation in these cells (Devin, Cook et al. 2000) and studies on RIP1 deficient Jurkat cells have found they have abrogated NF-κB signalling (Ting, Pimentel-Muinos et al. 1996). Binding studies show that IKK complex recruitment is unaffected in RIP1 deficient cells (Devin, Cook et al. 2000). Reconstitution studies have shown that RIP1 kinase activity is not required for TNFR1 mediated NF-κB activation (Lee, Shank et al. 2004).

NF-κB is activated by TNFR1 after the TNFR1-TRADD-RIP1 complex has assembled and become ubiquitinated with Lys-63 polyubiquitin chains (Ea, Deng et al. 2006). The IKK complex and the IKK activating complex TAK1-TAB2-TAB3 are recruited to the activated receptor through the ubiquitin binding domains and undergoes phosphorylation, perhaps by RIP1, and lysine 63 ubiquitination (Fan, Yu et al. 2010), which activates TAK's kinase domain and leads to the phosphorylation of IKKα and IKKβ. NF-κB is activated when activated IKKs phosphorylate the NF-κB inhibitory protein IκBα, leading to its proteasomal degradation. (An overview of TNF signalling is outlined in **Figure 1.5.**)

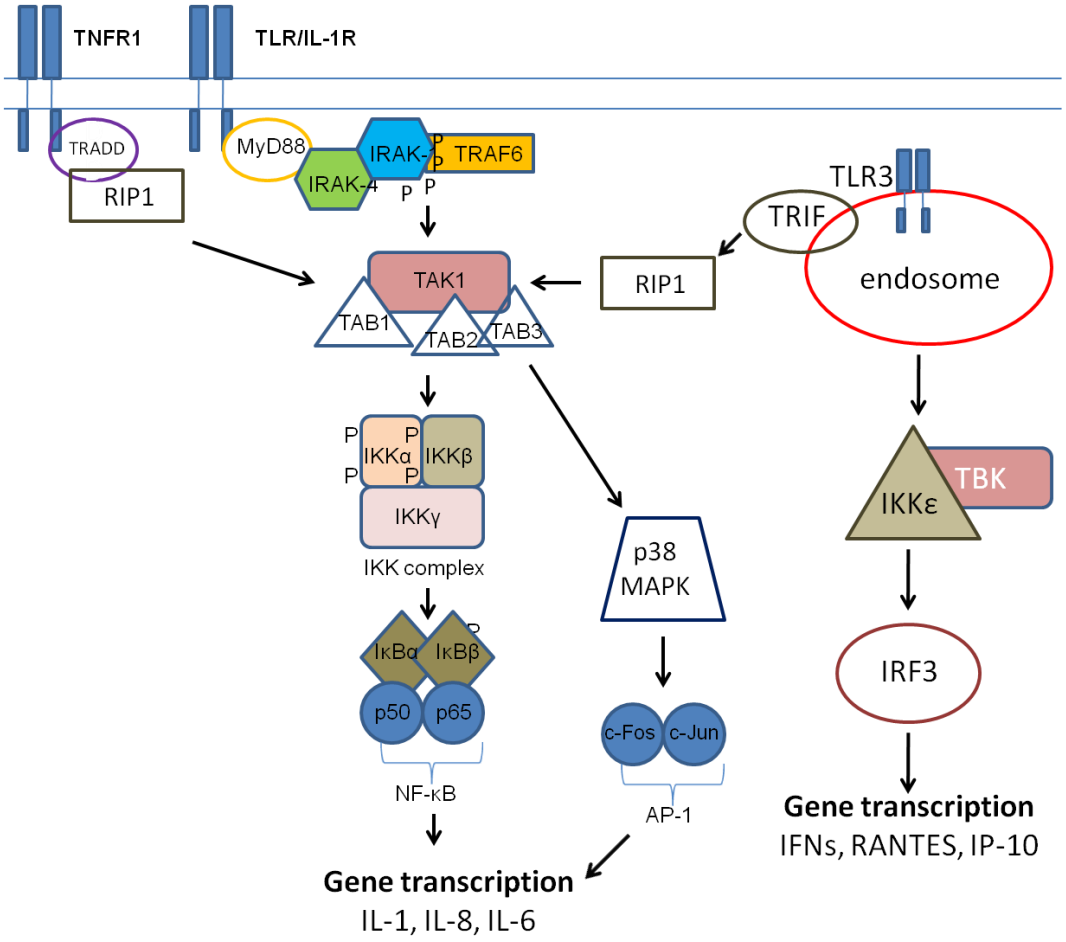


Figure 1.5: Inflammatory signalling pathways

Upon activation of TLR/IL-1R, MyD88 is recruited to receptor complex, which leads to phosphorylation (P) of IRAK-1 by IRAK-4 and the subsequent binding of TRAF6. IRAK-1: TRAF6 heterodimer leaves the receptor and binds TAK1 and its binding proteins, TAB1/2/3. TAK1 activates the IKK complex or MAPKs leading to the activation of NF-κB activation or AP-1 activation, respectively. Activation of these transcription factors leads to the upregulation of cytokines including IL-1, IL-8 and IL-6. TAK1 is also activated in response to TNFR1 stimulation by TNFα. Activated TNFR1 recruits TRADD and RIP1, leading to RIP1 Lys-63 ubiquitination and subsequent TAK1 activation. RIP1 is also involved in TLR3 signalling from the endosome, where it is recruited to the receptor complex via TRIF and this leads to NF-κB activation. TLR3 signalling also leads to the activation of a different transcription factor, IRF3, where TRIF activates the protein kinases TBK/IKKε that phosphorylate IRF3, leading to the activation of the transcription of cytokines such as the IFNs, RANTES and IP-10.

1.13 Ubiquitination and the post-translational modification of signalling

Phosphorylation is a well-described post-translational modification in signalling pathways, but more recently ubiquitination has been shown to be an influential mechanism for the regulation of signalling molecules. Ubiquitination is the process by which a 76 amino acid protein (8.5 kDa), ubiquitin, is covalently appended to a lysine (Lys) residue of target proteins. Three enzymes are required for different stages of ubiquitination; firstly ubiquitin is activated by E1 ubiquitin-activating enzymes, which catalyse the ATP-dependent thiolester bond formation between the ubiquitin C-terminal glycine and an E1 active site cysteine. Following activation, an E2 ubiquitin-carrier/conjugating protein (Ubc) binds high energy thiolester-ubiquitin from the E1 and delivers it to the E3 ubiquitin ligase. The E3 enzyme plays a key role in recognition and selection of proteins targeted for ubiquitination as it is responsible for ligating the C-terminus of ubiquitin to the ε-amino group of a lysine on the target protein to form an isopeptide bond (Moynagh 2008). There are three different types of E3 ubiquitin ligase, RING, F-box and HECT. The RING domain, and the closely related F-box type, E3s catalyse ubiquitination by transferring activated ubiquitin from the E2 enzyme whereas HECT type E3s transfer ubiquitin directly to the protein target (Pickart 2001).

Monoubiqutination is the addition of a single ubiquitin molecule, and since ubiquitin contains 7 lysine residues (K6, K11, K27, K29, K33, K48 and K63), they can also be linked together to form chains leading to polyubiquitination. The nature of the lysine linkage used to connect the polyubiquitin chains has great physiological implications.

Lysine-48 polyubiquitination acts as a tag for degradation of the target protein by the 26S proteasome, whereas Lys-63 linkage is linked to protein-protein interactions, trafficking and activation of kinases and phosphatases (Chen 2005; d'Azzo, Bongiovanni et al. 2005; Bhoj and Chen 2009). Polyubiquitination can also occur in a linear conformation whereby ubiquitins are linked together by a C-terminal lysine and an N-terminal methionine in a head-to-tail fashion (Kirisako, Kamei et al. 2006) and this type of ubiquitination has been to shown to be involved in NF-κB regulation by the RING type E3 ubiquitin ligases HOIL1 and HOIP (Tokunaga, Sakata et al. 2009). There have been reports of K11 linked polyubiquitin chains that have been linked mitosis and protein degradation (Matsumoto, Wickliffe et al. 2010); K27 linked polyubiquitin chains that have been associated with targeting Jun to lysosomes (Ikeda and Kerppola 2008); and K29 linked polyubiquitin chains that have been

shown to be involved in targeting Notch receptor for degradation (Chastagner, Israel et al. 2008), however these types of ubiquitination remain poorly understood. An overview of the ubiquitination process is outlined in **Figure 1.6**.

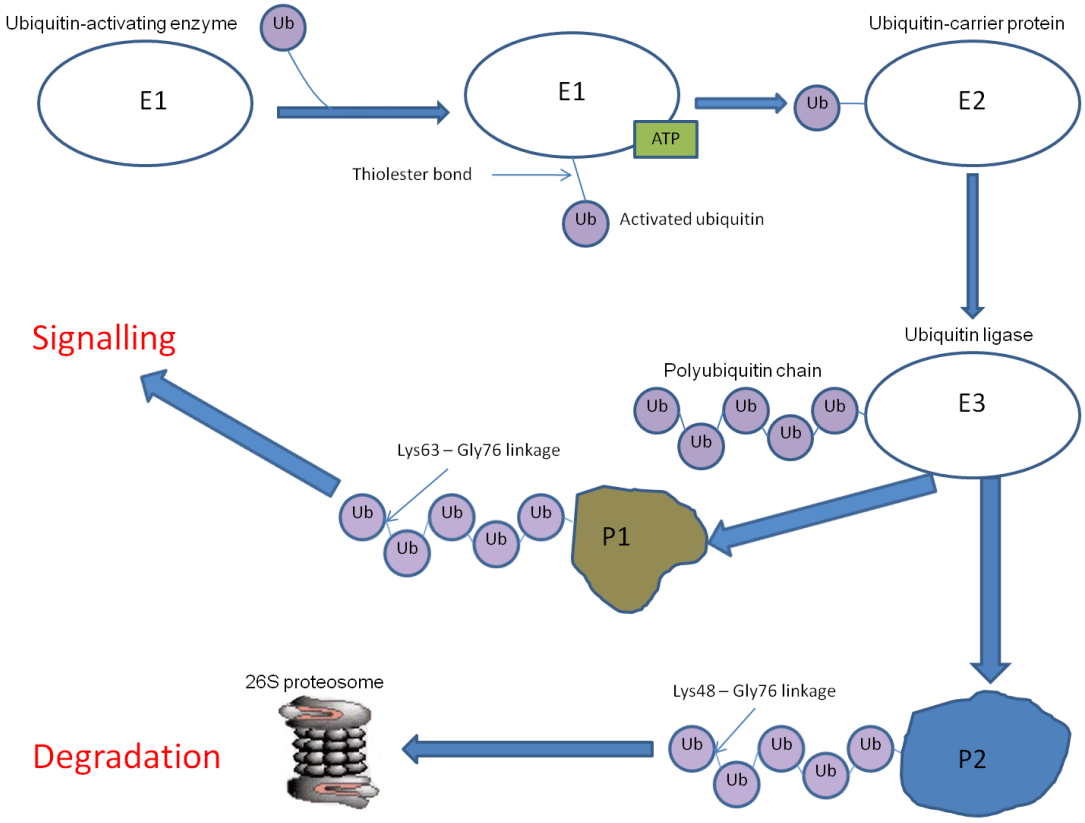


Figure 1.6: The ubiquitination pathway

The E1 ubiquitin-activating enzyme binds ubiquitin (Ub), which is activated during the formation of a high energy thiolester bond between Ub and E1. The E2 ubiquitin-carrier protein binds high energy Ub and delivers it to the E3 ubiquitin-ligase. Poly-Ub chains can form on E3 as the ε amino group of Lysine-48/63 bonds with a C-terminal Glycine76 on another Ub molecule. These poly-Ub chains can be ligated to the target protein (P1/P2) by the E3 ligase. The nature of the poly-Ub linkage determines the fate of the target protein, as Lys-48 poly-Ub chains target the protein for degradation and Lys-63 is involved in activation of downstream signalling molecules.

1.13.1 Ubiquitination in the TIR signalling pathway

TLR/IL-1R signalling pathways are highly regulated and the post-translational modification of ubiquitination plays a key role in this. E3 ubiquitin ligase function has been accredited to the RING, F-box, and HECT domains, which are found in many signalling molecules. TRAF6 is a key mediator of the TLR/IL-1R signalling pathway and contains an N-terminal RING domain which confers E3 ubiquitin ligase activity to this protein. As the IRAK1: TRAF6 dimer leaves the receptor complex, TRAF6 interacts with TIFA (TRAF6 interacting protein with FHA domain) that causes the oligomerisation and polyubiquitination of TRAF6 (Takatsuna, Kato et al. 2003). This Lys63-linked polyubiquitination is a prerequisite for TAK1 activation (Deng, Wang et al. 2000) and the chains are recognised by zinc finger domains inTAB2 and TAB3 (Kanayama, Seth et al. 2004). TRAF6 is also responsible for the Lys-63 polyubiquitination of IRAK-1 after IL-1 stimulation and this process regulates NEMO binding (Conze, Wu et al. 2008; Windheim, Stafford et al. 2008) and the production of unanchored Lys-63 polyubiquitin chains that have been shown to be involved in TAK1 and IKK activation (Xia, Sun et al. 2009).

IκB proteins, as noted earlier, are responsible for sequestering NF-κB in the cytoplasm until the IKK complex is stimulated, which leads to their degradation. This is accomplished by Lys48 ubiquitination of IκBα that targets the IκBs to the proteasome (Winston, Strack et al. 1999). This is carried out by an E2 of the UBD4/5 family (Chen, Parent et al. 1996) and the SCF-βTrCP E3 ligase (SKP1–CUL1–F-box ligase containing the F-box protein βTrCP). The βTrCP is the part that is responsible for binding to phosphorylated IκB through its WD40 repeats that recognises a 19 amino acid destruction motif in IκBα (residues 21-41) (Winston, Strack et al. 1999) and the RING containing SCF complex ligates Lys48-polyubiquitin chains onto IκBα conserved N-terminal lysine residues. Ubiquitinated IκB is subjected to selective degradation by the 26S proteasome, which releases NF-κB (Chen, Hagler et al. 1995).

Ubiquitination plays an important role in cell signalling and thus is tightly regulated. One way in which ubiquitination is regulated is by deubiquitinases (DUBs) which are enzymes that are involved in the removal ubiquitin chains from target proteins. One such DUB that has been linked to the negative regulation of NF-κB signalling is A20 (Jaattela, Mouritzen et al. 1996; Song, Rothe et al. 1996; Heyninck, De Valck et al. 1999). $A20^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibit prolonged NF-κB activation in response to IL-1 and TNFα stimulation as IKK complex is activated and IκBα is degraded for longer periods of time compared to that of wild type MEFs (Lee, Boone et al. 2000). Spleen cells from B cell-

specific A20 knockout ($A20^{CD19\text{-}KO}$) mice show enhanced IκBα degradation and p100 phosphorylation in response to the TNF superfamily ligand CD40, which suggests that A20 plays a negative regulatory role in both the canonical and non-canonical NF-κB signalling pathway (Tavares, Turer et al. 2010).

More recently, IRAKs have been shown to be subject to ubiquitination by a set of proteins known as Pellinos. These are the main focus of my research and so will be discussed independently (**See section 1.14**).

1.14 Pellino

Pellino was first discovered in *Drosophila melanogaster* as an interacting partner with Pelle, the *Drosophila* orthologue of IRAK (Grosshans, Schnorrer et al. 1999). Three mammalian Pellinos have since been discovered, Pellino 1, Pellino 2 and two splice variants of Pellino3, which are all highly conserved during evolution and share ~60% sequence homology to *Drosophila* Pellino.

1.14.1 Molecular function of Pellinos

The first mammalian Pellino, Pellino 1, was discovered in 2003 by Jiang *et al.* (Jiang, Johnson et al. 2003) and binding assays revealed that it interacts with IRAK1 and IRAK4 in an IL-1 dependent manner. In later years this attribute was found to span the entire Pellino family. Many over-expression studies have shown that Pellinos interact with other signalling components of the TLR/IL-1R pathway including TRAF6, TAK1, NF-κB-inducing kinase (NIK) and RIP1 (Jensen and Whitehead 2003; Jensen and Whitehead 2003; Jiang, Johnson et al. 2003; Chang, Jin et al. 2009) leading to the proposal of a scaffolding role. In 2006, Schauvliege *et al.* unravelled a new function for Pellinos by discovering a novel RING-like motif in the C-terminal. A traditional C3HC4 RING domain contains the 8 cysteine/histidine residues necessary to co-ordinate two zinc ions within the active site (Hanzawa, de Ruwe et al. 2001) and Pellinos were found to have a related Cys-Gly-His triplet sandwiched between two Cys-Pro-X-Cys motifs. This CHC2CHC2 RING is sufficient to confer the E3 ubiquitin ligase activity to Pellinos, which suggests they play a deeper role in signalling regulation than a scaffolding protein.

In support of this, *in vitro* studies have shown that Pellinos have the capability to catalyse a variety of polyubiquitin chains, depending on which E2 enzymes are available. Pellino1 can interact with an E2 UbcH3 to catalyse Lys48-linked polyubiquitination chains or

UbcH4/5a/5b to produce Lys48- and Lys11-linked chains (Ordureau, Smith et al. 2008). The E2 heterodimer UbcH13-Uev1a can work in conjunction with all Pellinos, which leads to the addition of Lys63-linked polyubiquitination chains and is the only type that has been seen at the cellular level (Xiao, Qian et al. 2008).

The significance of an intact IRAK kinase domain in the IRAK-Pellino interactions remains controversial. Some research shows that Pellino can bind to kinase-dead IRAK (Strelow, Kollewe et al. 2003; Butler, Hanly et al. 2007; Xiao, Qian et al. 2008) whereas others suggest the necessity of an active kinase (Jensen and Whitehead 2003; Schauvliege, Janssens et al. 2006). The former result may be an artefact from protein overexpression as *Drosophila* Pellino can only interact with Pelle with an active kinase domain (Grosshans, Schnorrer et al. 1999). Cellular experiments with naturally occurring kinase-dead forms of IRAK (IRAK-M, IRAK-2 and IRAK-1a splice variant) may be useful in clearing up this discrepancy. Co-expression studies, however, have revealed Pellinos to be a substrate for the IRAK kinase domain and this phosphorylation leads to enhanced E3 ubiquitin ligase activity resulting in intense Lys63-polyubiquitination of IRAK1 (Schauvliege, Janssens et al. 2006; Butler, Hanly et al. 2007; Xiao, Qian et al. 2008). The phosphorylation sites involved in the activation of Pellino1 by IRAK1 or IRAK4 have been mapped to 6 key serine/threonine residues, where phosphorylation of any of the key activating residues Ser76, Thr288 or Ser293, or the combined phosphorylation of Ser78, Thr80 and Ser82, leads to the activation of Pellino1 (Smith, Peggie et al. 2009). IRAK binding also results in reciprocal Pellino polyubiquitination by a separate but as yet unidentified E3 ubiquitin ligase, which leads to Pellino degradation (Butler, Hanly et al. 2007). This process may act as a negative feedback to terminate TLR/IL-1R signalling, as the Lys63-linked polyubiquitination of IRAKs is an important step to trigger downstream signalling events. Pellino1 has also shown to be sumoylated in a process similar to ubiquitination, which utilises E1 activating, E2 conjugating and E3 ligating enzymes. Sumoylation is the process by which a small ubiquitin-like modifier (SUMO) is appended to lysine residues of target proteins and has been shown to be appended to Lys202, Lys266, Lys295, Lys297 and Lys303 of Pellino1, which partially overlap with the ubiquitination sites of Lys169, Lys202 and Lys266 (Kim, Sung et al. 2011). Sumoylation has been linked to numerous essential cellular processes including nucleo-cytoplasmic trafficking, cell-cycle regulation, maintenance of genome integrity and transcription (Melchior 2000; Johnson 2004; Hay 2005; Kim and Baek 2006); however the functional significance of Pellino1 sumoylation is unclear.

The crystal structure of Pellino2 minus the RING domain was solved to 3.3 Å and revealed a forkhead-associated (FHA) domain within the N-terminal region (**Figure 1.7**). This FHA contains an unusual appendage named the wing domain (Lin, Huoh et al. 2008). Other RING E3 ubiquitin ligases have shown to contain the FHA domain including Chfr and RNF8 (Kang, Chen et al. 2002; Huen, Grant et al. 2007). FHA domains have been shown to mediate the binding of phosphothreonine domains and facilitate the binding of Pellino to phosphorylated IRAK (Acton, Dahlberg et al. 1996).

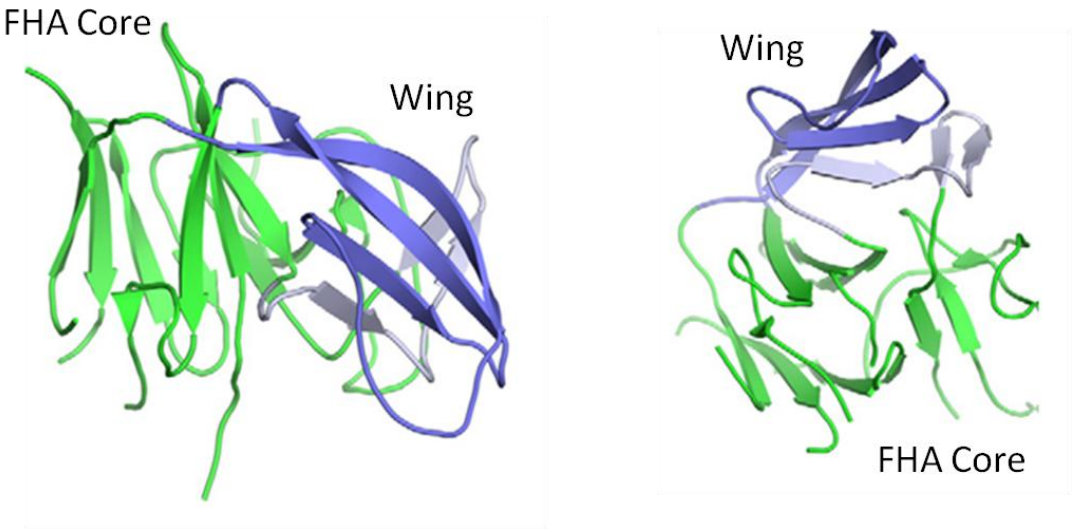


Figure 1.7: Crystal structure of Pellino2 FHA domain

The crystal structure of Pellino2 without the RING domain has been solved to 3.3 Å and can be seen as ribbon diagrams in two orientations. The forkhead-associated (FHA) domain is made up of 11 β-strands and is represented in green and the unique wing domain, which is made up of 6 β-strands, is represented in blue (Lin, Huoh et al. 2008).

1.14.2 Physiological function of Pellino1

The physiological importance of Pellino proteins in TLR/IL-1R signalling remains elusive and current models are based on overexpression models and contradicting data. The elucidation has become increasingly more complex with the emergence of specific niches for each Pellino. Pellino1 has been shown to be important in IL-1-dependent NF-κB activation and subsequent IL-8 production but knockdown of Pellino1 in transfected HEK293 cells did not affect TNFα-induced NF-κB activation (Jiang, Johnson et al. 2003). Unlike other Pellinos, Pellino1 is not involved in the regulation of MAPK pathways (Jensen and Whitehead 2003; Butler, Hanly et al. 2005).

Pellino 1 could prove to be a potential target to down regulate inflammation in the treatment of inflammatory diseases such as asthma and COPD. Pellino1 has already been shown to be a target for endogenous anti-inflammatory molecules including the anti-inflammatory cytokine transforming growth factor-β1 (TGF-β1) and MicroRNA-21. TGF-β1 induces its anti-inflammatory effects by releasing Smad6 and Smad7 in the cytoplasm. Smad6 and Smad7 are collectively known as the inhibitory-Smads (I-Smads) and act to negatively regulate signalling molecules controlling inflammation. I-Smads have been shown to sequester Pellino1 by binding to it through their MH2 domains and this inhibits the IRAK: Pellino: TRAF6 complex thus restricting NF-κB activation (Choi, Lee et al. 2006; Lee, Kim et al. 2010). MicroRNAs control expression of target genes by binding to the 3' untranslated region (UTR) of mRNAs to down regulate their expression. MicroRNA21 was shown to be upregulated during proliferative phases of liver generation and this coincided with a reduction in Pellino1 and IL-6 release. MicroRNA21 was shown to bind to the 3'UTR of Pellino1 and overexpression of this molecule inhibited NF-κB signalling (Marquez, Wendlandt et al. 2010).

More recently with the generation of the Pellino1 knockout mouse, it has been suggested that it is involved in TLR3 signalling (Chang, Jin et al. 2009). Pellino1 deficient mice develop normally but are resistant to LPS-induced toxic shock and show impaired B-cell proliferation and induction of co-stimulatory molecules CD86 and MHC class II molecules. In contrast to previous studies, Pellino1 deficient MEFs showed preserved IL-1 signalling, however had impaired responses to the TLR3 agonist and viral mimic poly(I:C) (Chang, Jin et al. 2009). This study also showed that Pellino1 was capable of binding to and ubiquitinating RIP1 and this was suggested as a potential Pellino1 target in TLR3 signalling (Chang, Jin et al. 2009). Pellino1 has also been linked to the transcription factor IRF3, where TBK and IKKε

phosphorylate Pellino1 leading to the activation of its E3 ligase activity and this was dependent on the presence of IRF3 (Smith, Liu et al. 2011).

1.14.3 Pellino1 in disease

Kawasaki disease (KD) is an acute, self-limiting vasculitis that occurs predominantly in infants and young children. Approximately 15-25% in untreated and 3-5% of treated children develop coronary artery aneurysms (Newburger, Takahashi et al. 1986; Durongpisitkul, Gururaj et al. 1995; Kato, Sugimura et al. 1996) and is the leading cause of acquired heart disease among children in developed countries. A recent genome-wide association study found that mutations in the *Peli1* gene in the 2p13.3 region is associated with the susceptibility to coronary artery aneurysms caused by KD (Kim, Hong et al. 2011).

A viral form of Pellino has been identified in the open reading frame (ORF) of the genome of *Melanoplus sanguinipes entomopoxvirus* (MsEPV). This viral Pellino (v-Pellino) shows poor overall sequence homology (15.6%) with mammalian Pellinos, however are structurally related. Like mammalian Pellinos, v-Pellino contains a FHA domain and can interact with IRAK-1, however unlike their mammalian counterparts v-Pellino is unable to modify IRAK-1. V-Pellino may act as a mammalian Pellino antagonist and is capable of attenuating NF-κB activation in response to LPS (Griffin, Mellett et al. 2011).

1.14.4 Physiological role of Pellino2

The expression pattern of murine Pellino2 was found to be highly tissue specific, where transcripts were predominant in liver, skin and testis but barely detectable in thymus or spleen. The expression pattern of human Pellino2 has yet to be published. The function of Pellino2 is ambiguous due to the contradicting nature of the minor amount of data available. Jensen *et al* overexpressed Pellino2 in HEK293 cells and reported that it was involved in activating the cJun, Elk-1 and CHOP transcription factors via the MAPK sub-pathway of TLR/IL-1R signalling (Jensen and Whitehead 2003). In contrast, Strelow *et al* overexpressed Pellino2 in HEK293R1 (HEK stably overexpressing IL-1R) and found no reproducible effects on NF-κB, JNK or Erk (MAPKs) but a consistent increase in luciferase activity in reporter assays that were independent of specific luciferase genes or specific transcription factor binding sites. It was suggested that Pellino2 acts as generic link between TIR signalling and transcriptional machinery (Strelow, Kollewe et al. 2003). Moving away from overexpression studies and toward *in vivo* or *ex vivo* experiments may be able to clear up such discrepancies.

Similar to Pellino 1, Pellino 2 has recently been show to be a target for anti-inflammatory molecules according to a study that identified B-cell lymphoma 10 (BCL10), an activator of NF-κB, as a binding partner with Pellino2. Pellino2 is a target of suppressor of cytokine 3 (SOC3) that restricts Pellino2: BCL10 interactions leading to an anti-inflammatory response (Liu, Dong et al. 2004).

1.14.5 Physiological role of Pellino3

Human Pellino1 and Pellino2 share over 80% amino acid homology, but only 70% with Pellino3, which contains a unique N-terminal stretch of 27 amino acids. Two splice variants of Pellino3 have been discovered, 3a and 3b, differing only in exon 3 where Pellino3b has a 24 amino acid deletion (Jensen and Whitehead 2003). Pellino3 is ubiquitously expressed at high levels in human brain, heart and testis and in lower levels in kidney, liver, lung, placenta, small intestine, spleen and stomach. Both Pellino 3a and 3b were present in the above tissues, with the exception of the lung, where only 3b was detected (Jensen and Whitehead 2003). Early research suggests Pellino3 plays a role in the MAPK pathways that activate the AP-1 family of transcription factors (c-Jun and Elk-1) and has no effect on NF-κB activation (Jensen and Whitehead 2003). This is supported by Butler *et al* (2005), who found Pellino3 to activate the p38 MAPK pathway, which leads to the activation of the cAMP response element binding protein (CREB) transcription factor (Butler, Hanly et al. 2005). Pellino1 and Pellino2 were unable to activate this pathway, (Jensen and Whitehead 2003) emphasising the lack of redundancy in the Pellino family.

Recent findings identify a role for Pellino3b as a negative regulator for IL-1-induced NF-κB activation by regulating IRAK degradation (Xiao, Qian et al. 2008). IRAKs can be ubiquitinated through both Lys-48 and Lys-63 polyubiquitin chains but only one type can be added to the IRAK ubiquitination site (Lys-134). Pellino3b mediates Lys-63-polyubiquitination of IRAK that inhibits Lys48-polyubiquitination-induced proteasomal degradation of IRAK (Xiao, Qian et al. 2008). Previous studies show that following IL-1 treatment IRAKs are recruited to the receptor, subsequently ubiquitinated and eventually degraded and that this degradation is essential for TAK1-dependent NF-κB activation (Yao, Kim et al. 2007). IRAK degradation at the membrane is accompanied by TAK1:TAB1:TAB2 release from the preassembled membrane-bound complex II (Jiang, Ninomiya-Tsuji et al. 2002) and so it was postulated that Pellino3b acts as a negative regulator of IL-1 induced NF-κB activation by blocking IRAK degradation and therefore TAK1 translocation (Xiao, Qian et al. 2008).

1.15 Hypothesis and Aims

The direct function of each Pellino is still yet to be elucidated but is an active area of research, especially considering its intimate relationship with inflammation. Pellino1 function remains controversial and the target and consequence for its E3 ubiquitin ligase activity is largely unclear.

I hypothesise that Pellino proteins play an important role in inflammation. More specifically, I hypothesis that:

1. Pellino expression is regulated in response to pro-inflammatory stimuli.
2. Pellino1 regulates TLR/IL-1R signalling in airway epithelial cells.
3. Pellino1 regulates inflammatory responses to viral pathogens.

The main aims of my thesis were threefold:

1. To investigate the expression and regulation of the Pellino family in human cell lines and primary human cells.
2. To create stable Pellino1 knockdown human cell lines, HeLa and the lung airway epithelial cell line BEAS 2B.
3. Use Pellino1 knockdown primary bronchial epithelial cells to investigate the role of Pellino1 in TLR/IL-1R signalling in response to viral and other pro-inflammatory stimuli.

2 Chapter 2 - Materials and methods

2.1 Materials

Reagents were purchased from Sigma-Aldrich (Poole, UK) or Invitrogen (Paisley, UK), except where specified. Foetal calf serum (FCS) was purchased from Promocell, Heidelberg, Germany. Recombinant human IL-1β and TNFα were purchased from Peprotech, New Jersey, USA. Poly(I:C) was purchased from Invivogen (Toulouse, France). OptiPrep^{TM} density gradient was purchased from Axis shield (Oslo, Norway). MACS LS columns were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Cell culture flasks were purchased from Nunc (Thermo Fisher Scientific, Loughborough, UK) and cell culture plates were purchased from Costar (Sigma-Aldrich). RT-PCR primers were ordered from Eurogentec (Southampton, UK) unless otherwise stated. Matched ELISA antibody pairs were purchased from R&D Systems (Abingdon, UK).

2.2 Mammalian cell culture

Cell lines and primary cells were used to investigate the expression pattern of Pellino proteins, to create stable Pellino1 stable cell lines and to ascertain Pellino1 function. Cells were thawed from frozen stocks (stored at -150$^{\circ C}$) and resuspended in cell culture medium. Cells were grown in 75 cm^2 culture flasks in a humidified 37°C incubator with 5% CO_2 and passaged as they reached 80-90% confluence (approximately every 3-4 days). All cells were purchased from the American Type Culture Collection (Manassas, VA, USA) unless otherwise stated.

2.2.1 Maintenance of HeLa

HeLa cells were originally derived from immortalised cervical cancer cells. These cells are maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % heat-inactivated FCS, 1 % penicillin and 1 % streptomycin. Cells were passaged twice weekly using Trypsin-EDTA (0.05% v/v trypsin and 0.5 mM EDTA).

2.2.2 Maintenance of HeLa-Ohio

HeLa-Ohio cells were used in the production of rhinovirus1B due to their uniform susceptibility for rhinoviral infection. HeLa-Ohio cells were maintained in DMEM supplemented with 10 % heat-inactivated FCS, 2.5 % HEPES buffer solution and 1 % sodium bicarbonate solution. These cells were passaged twice weekly using Trypsin-EDTA (0.05% v/v trypsin and 0.5 mM EDTA).

2.2.3 Maintenance of HEK cells

Human embryonic kidney (HEK) 293FT cells were maintained in DMEM supplemented with 10 % heat-inactivated FCS, 0.1 mM non-essential amino acids (NEAA), 2 mM L-Glutamine, 1 % penicillin, 1 % streptomycin and 500 µg/ml geneticin and passaged using trypsin-EDTA.

2.2.4 Maintenance of BEAS-2B

BEAS-2B cells are an immortalised lung airway epithelial cell line that was maintained in RPMI 1640 supplemented with 10 % heat-inactivated FCS, 1 % penicillin and 1 % streptomycin. All cells were incubated at 37° with 5 % CO_2. These cells were passaged using cell dissociation solution.

2.2.5 Maintenance of primary bronchial epithelial cells

Primary Bronchial Epithelial Cells (PBECs) were isolated from healthy human volunteers and purchased from PromoCell. These cells were maintained in serum-free Airway Epithelial Cell Growth Medium (PromoCell) supplemented with 0.004 ml/ml bovine pituitary extract, 10 ng/ml recombinant human epidermal growth factor, 5 µg/ml recombinant human insulin, 0.5 µg/ml hydrocortisone, 0.5 µg/ml adrenaline, 6.7 ng/ml triiodo-L-thyronine, 10 0.5 µg/ml human transferrin and 0.1 ng/ml retinoic acid. Media was replaced every 2-3 days and cells were passaged every 7-12 days using PromoCell® Detach kit.

2.2.6 Mycoplasma testing

Cell culture medium was tested monthly for the presence of mycoplasma using the EZ-PCR mycoplasma test kit, which amplifies mycoplasma specific DNA by PCR (Geneflow, Staffordshire, UK). This kit was carried out according to the manufacturer's instructions.

2.3 Isolation and purification of leukocytes

Peripheral venous blood was taken, with informed consent, from healthy volunteers in accordance with a protocol approved by the South Sheffield Local Research Ethics Committee. Human neutrophils and peripheral blood mononuclear cells (PBMCs) were purified by centrifuging whole blood that had been treated with the anticoagulant, 3.8 % Sodium Citrate (4.4 ml per 35.4 ml blood), at 1200 rpm (270 g) for 20 minutes. The upper platelet-rich plasma layer was removed and spun for a further 20 minutes at 2000 rpm to obtain the platelet poor plasma (PPP). Dextran T500 (6% w/v) in saline (6 ml) was added to the cell-rich lower phase from whole blood and 0.9 % saline was added to the volume of 50 ml. Following gentle mixing by inversion, the erythrocytes were left to sediment for 30

minutes. The supernatant containing all leukocytes was removed and pelleted by spinning at 1000 rpm for 6 minutes.

Leukocytes were separated according to density by centrifugation over an OptiPrep™ gradient. The gradient was achieved by resuspending leukocytes in 6 ml Hank's buffered salt solutions (HBSS) supplemented with 20 % PPP and adding 4 ml of OptiPrep™. After gently mixing by rotation, 10 ml of a 1095 density solution (8.036 ml HBSS, 20 % PPP and 3 ml OptiPrep™) was gently overlaid over the 10 ml cell solution. Above this, 10 ml of a 1080 density solution (10.435 ml HBSS, 20 % PPP and 3 ml OptiPrep™) was overlaid followed by a 10 ml layer of HBSS plus 20 % PPP. The gradient was spun at 1978 rpm (700 g) without brake, for 30 minutes at room temperature, which yields three populations of cells. Remaining erythrocytes were found at the interface between the cell rich layer and 1095. Neutrophils populate the 1095 – 1080 interface and PBMCs were found at the top of the 1080 layer. These cells were removed by Pasteur pipette in to separate clean tubes and cell numbers were determined using a haemocytometer.

2.3.1 Negative Magnetic Selection of Neutrophils

Peripheral blood neutrophils that have been separated by OptiPrep™ still show residual contamination from other leukocytes. In order to obtain a neutrophil sample that is over 98% pure, they must be passed over a negative magnetic selection column that depletes magnetically labelled non-neutrophils by retaining them in a column held within a magnetic field.

(i) Neutrophils isolated from OptiPrep™ separation gradient were pelleted by centrifuging at 1000 rpm for 6 minutes and resuspended in 1 ml column buffer (HBSS + 2 % FCS) per million neutrophils.

(ii) Sample was incubated with 70 µl of antibody cocktail (per 100 million cells) for 15 minutes (monoclonal antibodies to human cell surface antigens: CD36, CD2, CD3, CD14, CD19, CD56, Glycophorin A that label non-neutrophils) (Stem Cell Technologies, Grenoble, France).

(iii) Magnetic colloid beads (Stem Cell Technologies) were vortexed and 50 µl (per 100 million cells) was added to sample and incubated for 15 minutes with occasional swirling.

(iv) Whilst colloid was settling, the MACS LC column was set up according to manufacturer's instructions and it was primed with 9 ml of column buffer. Flow-through was discarded.

(v) Sample was added to the column and topped up with column buffer until eluted volume reached 10 ml, which was collected in a clean 50 ml centrifuge tube.

(vi) After establishing ultra-pure neutrophil number using a haemocytometer, the cells were pelleted at 2000 rpm for 2 minutes and resuspended in RPMI 1640 + 10 % FCS + 1 % penicillin + 1 % streptomycin.

2.3.2 Negative Magnetic Selection of Monocytes

Monocytes only make up approximately 10 % of PBMCs, the rest are made up of T and B cells and natural killer (NK cells). In order to purify monocytes from these and other contaminating erythrocytes and granulocytes, monocytes were purified from PBMCs by negative magnetic selection. The Monocyte Isolation Kit II (Miltenyi Biotec) provides a biotin-conjugated monoclonal antibody cocktail of CD3, CD7, CD16, CD19, CD56, CD123 and Glycophorin A that bind to non-monocytes. With the addition of a magnetic secondary label, anti-biotin monoclonal antibodies conjugated to MicroBeads^{TM}, the non-monocytes were retained in a MACS Column in the magnetic field of a MACS separator. Purified monocytes were washed through in the column buffer.

(i) PBMCs gained from OptiPrep^{TM} separation gradient were pelleted by spinning at 1000 rpm for 6 minutes and then resuspended in column buffer, which was made from 50 ml PBS, 200 µl 0.5 mM EDTA and 250 µl FBS. Thirty µl column buffer was used per 10^7 cells.

(ii) FcR blocking antibody (10 µl per 10^7 cells) and Antibody Cocktail (10 µl per 10^7 cells) were added to the cells with gentle mixing and then incubated at 4°C for 10 minutes.

(iii) A further 30 µl column buffer per 10^7 cells was added to the cells along with magnetic MicroBeads^{TM} (20 µl per 10^7 cells) and incubated for 15 minutes at 4°C.

(iv) The mixture was topped up with column buffer and centrifuged at 1000 g for 3 minutes to pellet cells. This pellet was resuspended in 500 µl column buffer and passed through a separation column that had been previously primed with 3 ml of column buffer.

(v) The cells were followed with 9ml of column buffer, all of which was collected in a clean 50 ml centrifuge tube.

(vi) The purified monocytes were pelleted by centrifuging at 2000 rpm for 2 minutes and were resuspended in 1 ml RPMI 1640 + 10 % FCS + 1 % penicillin + 1 % streptomycin. Cells were counted using a haemocytometer after a 1:10 dilution.

2.4 Reverse transcription PCR (RT-PCR)

2.4.1 Primer design

Specific PCR primers were designed for Pellino1, Pellino2, Pellino3a, Pellino3b and GAPDH (control) by finding the mRNA coding sequence on the NCBI database and inputting them into the PCR primer design programme MacVector™. Parameters were set to include the region of the mRNA where the primers were required and for primers of 18 to 24 nucleotides in length. Primer pairs were picked that had similar melting temperatures, span at least one intron-exon boundary and did not have several repeats of the same nucleotide as this can lead to mismatching during annealing. Primers were run through BLAST (basic local alignment search tool) to ensure specificity and ordered from Eurogentec (Southampton, UK). Primer sequences can be found in **Table 2.1**.

Target	Primers		Anneal (°C)	Product size (bp)
Pellino1	Fwd	5'-CCAAATGGCGATAGAGGAAGG-3'	55	376
	Rvs	5'-CATAAATCCGTGCTGTAAAGGGAG-3'		
Pellino2	Fwd	5'-CCCAATAAGGAGCCAGTGAAATAC-3'	57	242
	Rvs	5'-TCCACCACCACAGTCTGATTCC-3'		
	or			
	Fwd	5'-TCGTGTGCGACAGGAATGAAC-3'	59	367
	Rvs	5'-GGAGTATGAAAAAGCCCATCTGC-3'		
Pellino3a	Fwd	5'-TGTGAGGAAGGAGGTGAGGAAAC-3'	59	227
	Rvs	5'-ATGCTGTGCTGACCACGGTTAC-3'		
Pellino3b	Fwd	5'-TCATCGTCCTGGGCTACAATGG-3'	59	172
	Rvs	5-'ATGCTGTGCTGACCACGGTTACTC-3'		
GAPDH	Fwd	5'-ACTTTGGTATCGTGGAAGGAC-3'	50	420
	Rvs	5'-TGGTCGTTGAGGGCAATG-3'		

Table 2.1: RT-PCR Primers

RT-PCR primers were designed using MacVector™ and target specificity was verified using BLAST. The forward (Fwd) and reverse (Rvs) primer for each target is shown, along with the anneal temperature for the RT-PCR reaction and the expected product size.

2.4.2 RNA extraction and purification

RNA was extracted from cells using TRI Reagent. Cells were washed in PBS to remove any residual culture medium and up to 10 million cells were lysed in 1 ml of TRI Reagent. After incubating the sample for 5 minutes at room temperature to ensure complete lysis, 200 µl

of chloroform was added. After mixing vigorously, the solution was left to separate for 10 minutes at room temperature then centrifuged at 12000 g for 15 minutes at 4°C. This separates the solution in to three phases, the lower organic phase that contains protein, the interphase that contains DNA and the upper aqueous phase containing the RNA. The upper aqueous phase was transferred to a fresh tube. To this, 500 µl of isopropanol was added and after mixing vigorously, the solution was left to separate for 10 minutes at room temperature. RNA was pelleted by spinning the solution at 12000 g for 10 minutes at 4°C and after removal of the supernatant, the pellet was washed in 75% ethanol and centrifuged at 7500 g for 5 minutes at 4°C. The ethanol was removed to allow the RNA pellet to air-dry for approximately 10 minutes and then it was resuspended in 12 µl sterile H_2O. RNA yield and quality was assessed using the Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK).

2.4.3 cDNA synthesis

The high capacity cDNA reverse transcription kit (Applied Biosystems, California, USA) was used to convert 1 µg RNA into cDNA. The volume of RNA sample required was made up to 10 µl with sterile H_2O. To this, 2 µl 10X reverse transcriptase buffer, 0.8 µl 25X dNTP mix (100 mM), 2 µl 10X reverse transcriptase random primers, 1 µl MultiScribe™ Reverse Transcriptase, 1 µl RNase Inhibitor and 3.2 µl of nuclease-free sterile H_2O were added to the sample. To convert the RNA in to cDNA the master mix was incubated for 10 minutes at 25°C, followed by 120 minutes at 37°C and finally 5 minutes at 85°C in a HYBAID™ PCR express machine (Hybaid, Middlesex, UK).

2.4.4 Reverse transcription Polymerase chain reaction (RT-PCR)

Polymerase chain reaction (PCR) was carried out using GoTaq® Flexi DNA polymerase (Promega) by adding 5 µl 5X green GoTaq Flexi Buffer, 1.5 µl 25mM $MgCl_2$, 1 µl bulk dNTPs, 0.7 µl forward and reverse gene specific primers, 0.25 µl GoTaq DNA polymerase to 2 µl of cDNA sample. This master mix was made up to 25 µl with sterile H_2O and placed in the HYBAID™ PCR express machine. The samples were incubated at 95°C for 2 minutes for an initial denaturation period and then cycled through 95°C for 30 seconds to melt the double stranded DNA, 42-65°C for 1 minute (specific temperature needs to be optimised for each primer set that is based upon the melting temperature of the primers – see **Table 2.1**) to allow annealing of the primers to the specific region in the target gene and 72°C for 30 seconds to allow the heat-stable Taq polymerase to elongate from the primers. The final elongation step consists of 2 minutes at 72°C, which allows the Taq DNA polymerase to

rectify any unfinished amplicons. The samples were stored at -20°C until they were ready to be visualised by agarose gel electrophoresis.

2.5 Agarose gel electrophoresis

DNA molecules were separated and visualised by agarose gel electrophoresis. A 1.2 % gel was made by dissolving agarose in 1X TAE buffer (See **Appendix 1**) and boiling until the solution became clear. The agarose was left to cool to approximately 50°C before adding 0.3 µg/ml ethidium bromide with great care. The gel was poured in to the cast of the Easy Cast™ Electrophoresis System and a well-forming comb was added to the top of the gel. Once the gel had solidified, it was placed in the tank and submerged in 1X TAE buffer. The comb was removed and 5 µl of sample or HyperLadder IV DNA markers (Bioline) was loaded. The gel was run at 100 V for approximately 1 hour and visualised using CHEMI Genius^2 transilluminator (Geneflow) using the GeneSnap from Syngene software (Cambridge, UK).

2.6 Molecular cloning

2.6.1 Gel extraction of electrophoresed PCR products

PCR primers that lay to the 3' and 5' side of the TaqMan qPCR amplicons (**see section 2.7**) for Pellino1 and GAPDH were designed (**as outlined in section 2.4.1**), sequences of which are outlined in **Table 2.2**. These primers were used to amplify the target gene fragment using RT-PCR (**section 2.4.4**). The PCR products were electrophoresed (**section 2.5**) and DNA bands were visualised using a ultra-violet transilluminator. The correct sized band was excised from the gel with a sterile scalpel and weighed. DNA was extracted from the gel using QIAquick Gel extraction kit according to manufacturer's instructions. DNA was eluted in to 50 µl of sterile nuclease-free water and stored at -20°C.

Target	Primers		Anneal (°C)	Product size (bp)
Pellino1	Fwd	5'-CCAAATGGCGATAGAGGAAGG-3'	55	376
	Rvs	5'-CATAAATCCGTGCTGTAAAGGGAG-3'		
GAPDH	Fwd	5'-GTCGCCAGCCGAGCCACATC-3'	57	304
	Rvs	5'-GCCAGCATCGCCCCACTTGA-3'		

Table 2.2: RT-PCR Primers used for molecular cloning

Specific RT-PCR primers were designed using MacVector™ and target specificity was verified using BLAST. Primers were chosen that lay either side of the qPCR amplicon. The

forward (Fwd) and reverse (Rvs) primer for each target is shown, along with the anneal temperature for the RT-PCR reaction and the expected product size.

2.6.2 TOPO TA cloning of DNA fragments

DNA fragments were ligated in to pCR2.1-TOPO plasmid using the TOPO TA cloning kit (Invitrogen). Four µl of freshly isolated DNA product was incubated with 1 µl of salt solution (**Appendix 1**) and 1 µl of TOPO vector (**Appendix 1**) for 5 minutes at room temperature, which allowed ligation of the PCR product and the pCR2.1-TOPO plasmid. After ligation, reactions were placed on ice before they were used to transform competent cells.

2.6.3 Transformation of competent *E. coli*

One vial of One Shot Top 10 chemically competent cells (Invitrogen) was thawed on ice and 2 µl of ligation reaction was added to the cells. After mixing with a pipette tip, cells were incubated on ice for 15 minutes. Cells were heat-shocked by incubating in a 42°C waterbath for 30 seconds and then transferred immediately back to ice. 250 µl of SOC medium was added to the cells and shaken at 200 rpm at 37°C for 1 hour. Forty µl of 40 mg/ml X-gal was spread on to pre-warmed LB agar plates containing 100 µg/ml Kanamycin (added before LB agar had set) and 100 µl of each transformation was spread on to a plate and incubated at 37°C overnight.

2.6.1 Mini-prep analysis of plasmid DNA

Top10 competent cells overexpress the LacZ protein that contains a multiple cloning site in the middle of the β-galactosidase enzyme that will turn X-gal blue. Colonies that have incorporated the plasmid in the multiple cloning site will appear white as the β-galactosidase gene has been interrupted and therefore is not functional. Up to 10 white colonies were picked for analysis by PCR. Each colony was transferred to 2 ml of LB broth containing 100 µg/ml Kanamycin and incubated overnight on a shaking platform at 37°C. Cells were pelleted at 10,000 rpm for 3 minutes and the supernatant discarded. Plasmid DNA was extracted using QIAprep Mini prep kit (Qiagen, Crawley, UK) according to manufacturer's instructions. DNA was eluted in to 50 µl of sterile nuclease-free water and was quantified using the Nanodrop-1000® spectrophotometer. RT-PCR was carried out on each clone to test for the presence of the PCR insert.

2.7 Quantitative TaqMan PCR (qPCR)

Quantitative PCR was used to determine cellular levels of Pellino1, MyD88, IFNβ, IFNλ1 or IFNλ2/3 and was compared to that of the control gene GAPDH. Viral replication was also measured using qPCR using specific primers to part of the rhinovirus1B genome. Quantitative PCR involves gene-specific PCR primers that are recognised by Taq Polymerase, which amplifies part of the target gene. Within the two primers, a probe, which contains a fluorescent dye (FAM) at one end and a quencher (TAMRA) on the opposite end, binds to the cDNA or plasmid. FAM fluorescence is quenched by TAMRA when both molecules are in close proximity of each other. As the amplicon is amplified, the probe is degraded by the 5' to 3' exonuclease activity of Taq Polymerase, which releases FAM from the quenching activity of TAMRA. Fluorescence is directly proportional to amplification and can be read with a plate reader as the PCR reaction cycles. The ratio of fluorescence of sample is compared to a standard curve of known or arbitrary values to allow quantification of the mRNA in the sample. Cellular levels of target mRNA were determined by taking the ratio of the quantity of target mRNA compared to that of GAPDH control mRNA.

Primer probe sets were purchased from either Applied Biosystems (Pellino1, Hs00221035_m1; MyD88, Hs00182082_m1; and GAPDH, Hs00182082_m1) or Sigma-Aldrich (IFNβ, IFNλ1, IFNλ2/3 and RV1B – sequences can be found in **Appendix 1**). Primer-probe sets from Applied Biosystems were pre-mixed so 1 µl of this was added directly to 10 µl of 2x qPCR Mastermix (Eurogentec). Primers from Sigma were diluted to 300 nM for forward primers, 900 nM for reverse primers and 175 nM for probes and 1 µl of each was added to 10 µl of 2x qPCR Mastermix. One µl of cDNA sample (**see sections 2.4)** or standard **(see section 2.7.1)** was added to this mastermix, which was then made up to 20 µl with sterile water and sealed in a 384 PCR plate and centrifuged at 2000 rpm for 2 minutes. The plate was loaded in to the ABI 7900HT Fast Real-Time PCR System and the amplification cycle consisted of 50°C for 2 minutes, 95°C for 10 minutes followed by 40 repeats of 95°C for 15 seconds and 60°C for 1 minute. Samples were run in duplicate and data was analysed using SDS software version 2.2.1 and presented as ratio of target copy number compared to control copy number or as arbitrary units.

2.7.1 Quantitative PCR standards and Calculation of plasmid copy number

Sample concentrations were calculated from a standard curve of either arbitrary values (cDNA standards) or known copy number (plasmid standards). The IFNβ, IFN-λ1 and IFN-λ2/3 qPCR standard curve consisted of arbitrary values from RV1B infected BEAS-2B cells and the Pellino1 and GAPDH standard curves consisted of plasmids of known copy number **(see section 2.6 for cloning of standards)**.

For cDNA standards, the standard curve was initially set up by running 1:2, 1:4 and 1:10 serial dilutions of stock cDNA and the standard curve that exhibited the best range and intervals of Ct values was chosen. The cDNA standard curve for IFN-β used a 1:2 serial dilution and IFN-λ1 and IFN-λ2/3 used a 1:4 serial dilution. Plasmid standards were diluted down to 10^8 copies per µl before further 1:20 serial dilutions were carried out until the lowest standard contained 10^{-1} copies per µl.

Calculation of the copy number of the Pellino1 and GAPDH plasmids was carried out by calculating the molecular weight of each plasmid, based on the average molecular weight of a base pair being 650 Daltons. The number of moles of each plasmid was calculated by dividing the yield of plasmid by its molecular weight. Plasmid yield in each sample was quantified using the Nanodrop-1000® spectrophotometer and these values were used to define the number of molecules in the sample by dividing the number of moles multiplied by Avogadro's number ($6.02x10^{23}$) dividing this by the volume of the sample.

$$Moles = \frac{plasmid\ DNA\ yield\ (g)}{plasmid\ molecular\ weight\ (Daltons)}$$

$$Molecules\ (copies)/\ \mu l = \frac{(Moles\ x\ 6.02x10^{23})}{plasmid\ volume\ (\mu l)}$$

2.8 Western blot

Proteins were visualised using western blotting, where cell lysates are separated according to size by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Gel pouring, electrophoresis and transfer equipment were purchased from Bio-Rad.

(i) Whole cell lysates were made by resuspending cells in phosphatase lysis buffer (**see Appendix 1**) plus Triton-X plus 1 mM PMSF (added just before lysis) and Protease-inhibitor

cocktail (Calbiochem, Merck, Germany, diluted 1:100 in phosphatase lysis buffer, added just before lysis) and incubating on ice for 2 minutes followed by centrifugation at 10,000 rpm for 10 minutes at 4°C to remove insoluble material. The supernatant was transferred to a clean microcentrifuge tube, where the equivalent volume of hot SDS-PAGE lysis buffer was added and samples were boiled in a heatblock for approximately 5 minutes. Lysates were stored at -80°C until required.

(ii) SDS-PAGE gel apparatus was set up according to the manufacturer's instructions and the resolving gel mixture (**see Appendix 1**) was poured immediately after the TEMED was added. A gap of approximately 2.5 cm was left at the top for stacking gel and addition of a comb that forms the wells. The resolving gel was left to set at room temperature. Isopropanol was overlaid on the top of the gel to prevent evaporation. Once set, the isopropanol was washed away with water and then this water was removed. Stacking gel was overlaid over the resolving gel and a 10- or 15-well comb was added. The gels were then assembled in a running tank according to manufacturer's instructions and the combs were carefully removed. The tank was filled with 1 X running buffer (**see Appendix 1 section 3**).

(iii) Cell lysates were electrophoresed on an appropriate resolving gel (**see Appendix 1**) by loading up to 25 µl of lysate into each well and running at 80 V for approximately 10 minutes followed by 200 V (PowerPac 300, Bio-Rad, Hertfordshire, UK) until the dye front reached the bottom of the gel. Samples were run alongside 5 µl of ColorPlus Prestained Protein Ladder (New England BioLabs, Ipswich, Massachusetts, USA).

(iv) The proteins from the gel were transferred to a nitrocellulose membrane by sandwiching the gel and membrane between two pieces of filter paper and two sponges that were encased in the transfer casket. The transfer caskets were placed in the tank with the black side of the casket facing the back so the proteins will run from the gel onto the membrane (from negative to positive). Transfer buffer (**see Appendix 1**) was kept cold using ice packs within the tank and proteins were transferred for 70 minutes at 100 V. To examine transfer efficiency, proteins were crudely visualised by Ponceau S staining for 30 seconds, then the blot was blocked in blocking buffer (**see Appendix 1 section 3**) for 1 hour, to reduce non-specific antibody binding.

(v) After the membrane was washed for 5 minutes in PBS, the primary antibody was diluted in antibody buffer (**see Appendix 1 section 3**) and incubated with the membrane over night at 4°C on a rotating platform.

(vi) The following day, the membrane was washed of any unbound primary antibody by 3 X 10 minute washes in PBS-tween (**see Appendix 1 section 3**). After which, the relevant HRP-conjugated secondary antibody was diluted in antibody buffer and incubated with the membrane for 1 hour at room temperature on a rotating platform. Dilutions of primary and secondary antibodies can be found in **Table 2.3.**

(vii) After a further 3 X 10 minute washes in PBS-tween, the proteins were visualised using Enhanced Chemilluminescent Reagent (EZ-ECL^{TM} Chemiluminescence Detection kit for HRP, Geneflow). Blots were incubated in ECL for 2 minutes and exposed to photographic film (Hyperfilm, Amersham, GE Lifesciences, Buckinghamshire, UK) for the desired exposure time. Developed films were analysed by densitometry using NIH Image 1.62 analysis software (National Institute of Health, USA).

2.8.1 Cytosolic and membranous cell fractions

Cells were separated into cytoplasmic and membranous fractions using a light surfactant saponin. Saponin gently disrupts the plasma membrane to extract cytoplasmic proteins without solubilising membrane proteins. Cells were fractionated by resuspending in phosphatase lysis buffer without Triton-X plus 2% saponin (**Appendix 1** - 10 µl lysis buffer per 1 x 10^6 cells) and incubating at room temperature for 2 minutes. After which, cells were centrifuged at 10,000 g for 5 minutes at 4°C and the supernatant containing the cytoplasmic fraction was removed and added to the equivalent volume of hot SDS-PAGE lysis buffer and boiled in a heatblock for approximately 5 minutes. The pellet containing the membrane fraction was washed in lysis buffer without Triton-X or saponin to remove any residual cytoplasmic proteins before centrifuging at 10,000 g for 5 minutes at 4°C. The supernatant was discarded and cells were resuspended in phosphatase lysis buffer plus Triton-X (**Appendix 1** - 10 µl lysis buffer per 1 x 10^6 cells). After incubation for 2 minutes at room temperature, an equivalent volume of hot SDS-PAGE lysis buffer was added to the membranous fraction and boiled in a heatblock for approximately 5 minutes. Cell fractions were stored at -80°C until required for western blotting.

2.8.2 Stripping and re-probing blots

Blots were stripped of existing primary and secondary antibodies by washing the blot in PBS for 5 minutes, followed by water for 5 minutes and then soaked in 0.2 M sodium hydroxide for 8-10 minutes to remove any bound antibodies. The washing procedure was then repeated before incubating the blot in blocking buffer for 1 hour. New antibodies were added to the blot and developed as described above.

Target	Manufacturer	Species	Dilution	Mono- or Polyclonal
RIP1	BD Biosciences (Oxford, UK)	Mouse	1:1000	Monoclonal
IκBα	Santa Cruz Biotechnology (California, USA)	Rabbit	1:500	Polyclonal
NF-κB2 (p100/p52)	Cell Signalling Technology (Massachusetts, USA)	Rabbit	1: 500	Polyclonal
Actin	Sigma-Aldrich	Rabbit	1:5000	Polyclonal
Phospho-p38	Promega (Wisconsin, USA)	Rabbit	1:1000	Polyclonal
Total p38	Cell Signalling Technology	Rabbit	1:1000	
MyD88	Cell Signalling Technology	Rabbit	1:1000	Polyclonal

Table 2.3: Antibody dilutions

The antibodies used for immunoblotting and the species and dilutions used are outlined in the table. Each antibody was stored according to manufactures instructions.

2.9 Transient transfection of plasmid DNA

The plasmids pENTR™/H1/TO, pLenti4 and ViraPower™ Packaging Mix were transfected into cells using Lipofectamine 2000™ transfection reagent. Cells were plated in an appropriate cell culture vessel and grown to 90-95 % confluence. On the day of transfection, cells were washed twice in 1X PBS and antibiotic-free media was added to each well. Plasmid DNA and Lipofectamine 2000™ were made up to the appropriate volume with Opti-MEM® (**see Table 2.4**) in two separate microcentrifuge tubes and left to equilibrate for 5 minutes. The two solutions were combined and briefly vortexed before leaving the plasmid DNA to complex with the transfection reagent for 20 minutes at room temperature. This solution was then added to the cells and incubated at 37°C + 5 % CO_2 for overnight. An untransfected control, treated only with Opti-MEM® and a mock transfected control, treated only with transfection reagent in Opti-MEM® were also included. The next day, the cells were washed in 1X PBS and complete media was added to allow recovery. Cells were incubated for a further 24 hours at 37°C + 5 % CO_2 before harvesting.

Cell culture vessel	Surface area	Volume of plating medium	Volume of dilution medium	DNA	Lipofectamine
96-well	0.3 cm_2	100 µl	2 x 25 µl	0.2 µg	0.5 µl
24-well	2 cm_2	500 µl	2 x 50 µl	0.8 µg	2.0 µl
12-well	4 cm_2	1 ml	2 x 100 µl	1.6 µg	4.0 µl
6-well	10 cm_2	2 ml	2 x 250 µl	4.0 µg	10 µl
60-mm	20 cm_2	5 ml	2 x 0.5 ml	8.0 µg	20 µl
10-cm	60 cm_2	15 ml	2 x 1.5 ml	24 µg	60 µl

Table 2.4: Transfection of plasmid DNA using Lipofectamine 2000™

Lipofectamine 2000™ was used to transfect cells with plasmid DNA and the appropriate amounts of DNA and volumes of media and lipofectamine required for efficient transfection are outlined in the table.

2.10 Transient siRNA knockdown of Pellino1 and RIP1

Pellino1 or RIP1 was knocked down in BEAS-2B cells or PBECs using ON-TARGET plus SMARTpool™ (L-013814-01-0005 – Pellino1 and L-004445-00-0005 RIP1, Dharmacon, Thermo Fisher Scientific) system, which utilises 4 different target-specific siRNAs to lead to gene silencing. The siRNA was delivered in to the cells using Lipofectamine 2000™. To ensure specificity of phenotypical changes in knockdown cells, control cells were transfected with a non-targeting siRNA (D-001810-02-05, ON-TARGETplus Non-targeting siRNA #2 – Dharmacon) that controls for changes caused by the exposure of cells to siRNA.

Transfection of the siRNA was carried out by seeding cells in a 12-well plate (60,000 cells/well for PBECs and 200,000 cells/well for BEAS-2B cells) and culturing these cells at 37°C + 5 % CO_2 until they reached confluency. On the day of transfection, cells were washed twice in PBS and complete media was replaced with 800 µl RPMI 1640 supplemented with 10 % FCS but without antibiotics (BEAS-2B cells) or serum-free Airway Epithelial Cell Growth Medium (PromoCell®, for PBECs). The stock siRNA (20 µM) was diluted to a final concentration of 1 µM in Opti-MEM® and Lipofectamine was diluted 1 µl in 40 µl of Opti-MEM®. After equilibration at room temperature for 5 minutes, the two solutions were combined to allow the siRNA to complex with the transfection reagent for 20 minutes. Two hundred µl of the complexed siRNA was added to 800 µl of antibiotic-free media previously added to each well of the 12-well plate and the cells were incubated for 4 hours at 37°C + 5 % CO_2. Cells were then washed in 1X PBS 4 hours before adding 1 ml of complete growth media (BEAS-2B cells) or Airway Epithelial Cell Growth Medium supplemented with 10 ng/ml recombinant human epidermal growth factor, 5 µg/ml recombinant human insulin, 0.5 µg/ml hydrocortisone, 0.5 µg/ml adrenaline, 6.7 ng/ml triiodo-L-thyronine, 10 0.5 µg/ml human transferrin and 0.1 ng/ml retinoic acid but without bovine pituitary extract (PBECs) for recovery. Cells were incubated at 37°C + 5 % CO_2 for at least 18 hours before stimulating with proinflammatory agonists (carried out in complete growth medium for BEAS-2B or unsupplemented Airway Epithelial Cell Growth Medium for PBECs) or infection with RV1B (carried out in RPMI 1640 supplemented with 2% and penicillin 1% and streptomycin 1% for BEAS-2B cells or unsupplemented Airway Epithelial Cell Growth Medium for PBECs).

2.11 Rhinovirus 1B (RV1B)

2.11.1 RV1B culture

RV1B stocks were kindly provided by Sebastian Johnston and Michael Edwards (Imperial College, London) and RV1B was cultured by infecting a confluent monolayer of HeLa-Ohio cells in T175 tissue culture flasks. Cells were washed twice with 10 ml of infection media (DMEM supplemented with 4 % heat-inactivated FCS, 2 % HEPES buffer and 1% sodium bicarbonate) before being replaced with 5 ml of viral stock and 7.5 ml of infection media. Flasks were gently agitated for 1 hour at room temperature, after which, a further 12.5 ml of infection media was added and these cells were incubated overnight at 37°C + 5 % CO_2 or until approximately 90 % cytopathic effect was observed morphologically. Cells were lysed by freeze-thawing three times and cell debris was removed by centrifugation at 4000 rpm for 15 minutes. The resulting RV inoculum was filtered through a 0.2 µm filter and then stored in 5 ml aliquots at -80°C. RV1B culture and titration was carried out by C. Stokes and L. C. Parker in our laboratory.

2.11.2 RV1B titration in HeLa-Ohio

HeLa-Ohio cells were split, re-suspended in HeLa-Ohio infection media (see **section 2.11.1**) to give a concentration of $0.5X10^5$ cells/ml. One hundred and fifty µl of cells were placed in each well of a 96-well cell culture plate. Undiluted RV inoculum, or 10-fold serial dilutions were added to each well and the plate was incubated at 37°C + 5 % CO_2 for 4-5 days. Viral cytopathic effect (CPE) was determined using light microscopy and infected wells were counted on day 4-5. Tissue culture infective dose 50% ($TCID_{50}$)/ml value was determined by scoring the sum of positive wells and using the Spearman Karber formula, which is a non-parametric formula designed to determine the viral dose for a 50 % infection rate (**see Appendix 1 section 6**).

2.11.3 RV1B infection of BEAS-2B and PBECs

Rhinovirus 1B (RV1B) was used to infect a confluent layer of BEAS-2B or PBECs in a 12 well plate. Cells were serum starved by incubating in BEAS-2B infection media (**see section 2.10**) or PBEC normal recovery media (**see section 2.10**) overnight at 37°C + 5 % CO_2 before washing them with 1X PBS. Virus was filtered through a 30 KDa filter so RV1B virions were retained on the column and 250 µl of the flow-through was added to the cells as a filtrate control to ensure that the RV1B infection leads to the observed phenotype and not simply transfer of proinflammatory mediators from viral media. Two hundred and fifty µl of RV1B that had been inactivated with UV (1000 mJ/cm^2 for 10 minutes in a UV crosslinker) was

added to independent wells to show the importance of viral replication in cytokine release. Virus was diluted in infection media to the desired $TCID_{50}$ with infection media (BEAS-2B) or basal Airway Epithelial Cell Growth Medium (PBEC) and 250 µl of each dilution was added to cells and incubated at room temperature on a rotating platform for 1 hour. Virus was removed from cells and 1 ml of fresh infection (BEAS-2B) or basal Airway Epithelial Cell Growth Medium was added and cells were incubated for 24 hours at 37°C + 5 % CO_2. Supernatant was removed and analysed for cytokine release using ELISA (**see section 2.12**) and RNA from cells to determine interferon production by qPCR (**see section 2.7**).

2.12 Enzyme-linked immunosorbent assay (ELISA)

ELISAs were used to measure IL-8, IL-6, IP-10 or RANTES generation in Pellino1 and RIP1 knockdown BEAS-2B or PBECs that had been stimulated with TLR agonists or rhinovirus for 24 hours. Cell free supernatants were collected and stored at -80°C until required.

Coating antibody was diluted to the appropriate concentration (**Table 2.5**) in PBS coating buffer (**see Appendix 1 section 5**) and 100 µl was added to each well of a 96 well high binding plate and incubated overnight at room temperature. The plate was washed 3 times with wash buffer (**see Appendix 1 section 5**) using an EL_x50 auto strip washer (Bio-Tek Instruments Inc, Winooski, VT) then blocked in 1% Albumin (from chicken egg, Sigma-Aldrich) in PBS coating buffer for 1 hour at room temperature on an orbital shaker. After washing the plate as previously described, 100 µl of sample (diluted as required to ensure cytokine release falls within the standard range) or standard was added in duplicate to the plate. IL-8 and IL-6 standards ranged from 19.5 to 5000 pg/ml, IP-10 standards ranged from 15.6 to 4000 pg/ml and RANTES standards ranged from 39.1 to 10000 pg/ml and were prepared by 2-fold dilutions in wash buffer. The plate was incubated for 2 hours at room temperature on an orbital shaker, after which, the plate was washed as before and 100 µl of biotinylated detection antibody was added at the appropriate concentration (**Table 2.5**). The plate was then incubated for a further 2 hours at room temperature on an orbital shaker. The plate was then washed and 100 µl of streptavidin HRP, diluted 1 in 200 in wash buffer, was added to the plate and incubated in the dark on an orbital shaker for 20 minutes. After the final wash, the colour was developed using 100 µl of detection reagent (made up by combining subtrate A and B in a 1:1 ratio, R&D Systems) in the dark for 10 – 20 minutes. Once the standard curve has changed colour, the reaction was stopped using 50 µl 1 M sulphuric acid. The optical density (OD) of each well was read at 450 nm on a Opsys MR™ microplate reader (Dynex Technologies) using Revelation software version

4.25. The standard curve was plotted on a log/linear scale (Prism software version 5.0) and unknown samples were quantified against this standard curve. If the amount of cytokine in the sample was above the detection limit, then in subsequent ELISAs, the sample was diluted with wash buffer until it fell within the linear range of the standard curve.

Cytokine Antibody	Coating Antibody	Detection Antibody
IL-8	1.5 µg/ml	80 ng/ml
RANTES	2 µg/ml	20 ng/ml
IL-6	1 µg/ml	50 ng/ml
IP-10	2.8 µg/ml	0.28 µg/ml

Table 2.5: Concentration of antibodies used for ELISA

IL-8, IL-6, IP-10 and RANTES proteins were quantified using matched antibody pairs (R&D systems) for use in ELISA. Concentrations for coating and detection antibodies for each protein had previously been optimised in the lab and a summary of which is outlined in the table.

2.13 Cytometric Bead Array (CBA)

Production of multiple cytokines in a single sample was measured using a cytometric bead array (CBA) (BD Biosciences). Cell-free supernatants were prepared and stored at -80^{o}C until required. Cytokines were measured using CBA flex set multiplexed bead immunoassay (BD Biosciences) comprising antibodies to TNFα, GM-CSF, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, IL-10, IL-1β, IL-1α and CX3CL1/fractalkine.

Samples were analysed for cytokine production by incubating with beads of a known size that were coated in antibodies to the specific cytokine, which allowed them to be separated by flow cytometry. These sample-bound beads were detected by the addition of a detection antibody that is conjugated to phycoerythrin (PE) that provides a fluorescent signal in proportion to the amount of bound cytokine. Sample fluorescence is compared to that of a standard curve to determine the concentration of each cytokine. Samples and standards were analysed using a FACS Array (BD Biosciences).

2.14 Specific methods for the creation of Pellino1 and MyD88 knockdown stable cell-lines using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System

The BLOCK-iT™ Inducible H1 Lentiviral RNAi System (Invitrogen) was used to manufacture a replication-incompetent lentivirus that delivers a short hairpin RNA (shRNA) targeting Pellino1 or MyD88 into dividing or non-dividing mammalian cells for RNA interference (RNAi) analysis. Cells that had integrated the lentiviral plasmid into their chromosomes were selected and these cells were single-cell cloned to ensure homogeneous stable production of the shRNA, which leads to stable target gene knockdown.

2.14.1 Short hairpin RNA design

RNA interference describes the phenomenon by which dsRNA induces potent and specific inhibition of eukaryotic gene expression via the degradation of complementary messenger RNA (mRNA). In eukaryotic organisms, dsRNA produced *in vivo* or introduced by pathogens is processed into 21–23 nucleotide double-stranded short interfering RNA duplexes (siRNA) by an enzyme called Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases. Each siRNA then incorporates into a RNA-induced silencing complex (RISC), an enzyme complex that serves to target cellular transcripts complementary to the siRNA for specific cleavage and degradation. In addition to dsRNA, other endogenous RNA molecules including short temporal RNA (stRNA) and micro RNA

(miRNA) have been identified and have been shown to be capable of triggering gene silencing. Short hairpin RNA (shRNA) is a simplified, artificially-made RNA that is capable of silencing genes using the components of the miRNA and siRNA pathways.

Short hairpin RNA usually consists of a short nucleotide sequence ranging from 19–29 nucleotides derived from the target gene, followed by a short spacer sequence of 4–15 nucleotides, which forms the loop, and ends in a 19–29 nucleotide sequence that is the reverse complement of the initial target sequence. This allows the molecule to undergo internal base pairing and forms a stem-loop hairpin structure that is recognised by dicer and cleaved into siRNA. The use of shRNA in the creation of stable knockdowns is beneficial as they are more stable than siRNA, which assists the longevity of the knockdown, and they are able to be transcribed by RNA Polymerase III. RNA Polymerase III transcribes a limited number of genes, many of which are small stable RNA molecules that are involved in RNA processing. The beneficial properties of this polymerase are that the transcription initiates and terminates at fairly precise points and there is little addition of unwanted 5' and 3' sequences to the RNA molecule.

Single-stranded oligos that target Pellino1 or MyD88 were designed using BLOCK-iT^{TM} RNAi Designer (Invitrogen), which designed oligos that were 19-29 nucleotides in length; specific to the target genes; avoid runs of 4 or more of the same nucleotide; and had a low G/C content (below 50%). The sets of primers designed for Pellino1 and MyD88 can be found in **Table 2.6**.

The single-stranded oligos were annealed together by mixing 5 µl of 200 µM top strand oligo and 5 µl of 200 µM bottom strand oligo with 2 µl of 10X annealing buffer (**Table 2.7**) and 8 µl of RNAse-free water. This solution was heated to 95°C for 4 minutes and then left to cool at room temperature for 5-10 minutes. The resulting double-stranded oligo (dsOligo) was then cloned into pENTR/H1/TO.

Primers	
MyD88	
Top	5’- CACCGCACCTGTGTCTGGTCTATTGCGAACAATAGACCAGACACAGGTGC-3’
Bottom	5’- AAAAGCACCTGTGTCTGGTCTATTGCGAACAATAGACCAGACACAGGTGC-3’
dsOligo	5’-CACCGCACCTGTGTCTGGTCTATTGCGAACAATAGACCAGACACAGGTGC-3’ 3’-CGTGGACACAGACCAGATAACGCTTGTTATCTGGTCTGTGTCCACGAAAA-5’
Pellino1	
Top	5’- CACCGGGTTCAACACACTAGCATTTCGAAAAATGCTAGTGTGTTGAACCC-3’
Bottom	5’- AAAAGGGTTCAACACACTAGCATTTTTGCAAATGCTAGTGTGTTGAACCC-3’
dsOligo	5’-CACC GGGTTCAACACACTAGCATTTCGAAAAATGCTAGTGTGTTGAACCC-3’ 3’-CCCAAGTTGTGTGATCGTAAAGCTTTTTACGATCACACACAACTTGGGAAAA-5’

Table 2.6: shRNA design for Pellino1 and MyD88

The shRNA targeting Pellino1 and MyD88 was designed using the BLOCK-iT™ RNAi Designer (Invitrogen). The table shows the linker sequence (red), the sense sequence (blue), the linker sequence (green) and the anti-sense sequence (pink). The resulting double-stranded oligo (dsOligo) is shown underneath.

Reagent	Composition
pENTR™/H1/TO vector, linearised	0.75 ng/µl plasmid DNA in: 10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0
10X Oligo Annealing Buffer	100 mM Tris-HCl, pH 8.0 10 mM EDTA, pH 8.0 1 M NaCl
5X Ligation Buffer	250 mM Tris-HCl, pH 7.6 50 mM $MgCl_2$ 5 mM ATP 5 mM DTT 25% (w/v) polyethylene glycol-8000
T4 DNA ligase	1 (Weiss) U/µl in 10 mM Tris-HCl, pH 7.5 50 mM KCl 1 mM DTT 50% (v/v) glycerol

Table 2.7: Reagents for manufacturing of the entry clone pENTR™/H1/TO

The table outlines the composition of the reagents supplied in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System used in the manufacture of the double-stranded oligos encoding a shRNA that is used to target specific genes for silencing via the RNAi pathway and its subsequent cloning into the pENTR™/H1/TO plasmid.

2.14.2 Cloning of pENTR™/H1/TO

The resulting dsOligo was cloned into the entry vector pENTR™/H1/TO (plasmid map and features can be found in **Appendix 1**) by adding 4 µl of 5X ligation buffer, 2 µl pENTR™/H1/TO (0.75 ng/µl), 2 µl of 5 nM dsOligo and 11 µl of DNase/RNase-free water into a microfuge tube. Once the rest of the ligation reaction was set up, 1 µl of 1U/µl T4 DNA ligase was added to catalyse the joining of the dsOligo into the pENTR™/H1/TO plasmid. The reaction was mixed well by pipetting and left to incubate for 5 minutes at room temperature. 2 µl of this ligation reaction was then transformed into One Shot® TOP10 Chemically Competent *E. coli* by incubating together on ice for 15 minutes and then heat-shocking the cells at 42°C for 30 seconds. The tube of cells was immediately transferred to ice where 250 µl of S.O.C medium (**Appendix 1**) was added and this was then shaken at 200 rpm at 37°C for 1 hour. After this incubation time, 50 µl or 100 µl of transformants were spread onto separate pre-warmed LB agar plates (**Appendix 1**) containing 50 µg/ml kanamycin. 5 antibiotic resistant clones were picked and grown overnight in LB broth (**Appendix 1)** containing 50 µg/ml kanamycin. Plasmid DNA was isolated using MiniPrep Kit (Qiagen) according to manufacturer's instructions and were sequenced to ensure the presence and correct orientation and sequence of the dsOligo insert.

2.14.3 Cloning the pLenti4/BLOCK-iT™ expression construct

The plasmid that exhibited the best knockdown capability for Pellino1 or MyD88 was cloned into the destination vector pLenti4/BLOCK-iT™-DEST vector. This vector contains elements required to allow packaging of the expression construct into virions and an antibiotic resistance marker to allow selection of stably transduced cell lines (**Appendix 1**)**.** The lentivirus is able to enter the target cell, where the viral RNA is reverse-transcribed, actively imported into the nucleus and stably integrated into the host genome. Once the lentiviral construct has integrated into the genome, the shRNA of interest is constitutively expressed and the target gene is knocked down using the RNAi pathway. The virus was manufactured by initially performing a ligation reaction between the pENTR™/H1/TO entry clone and the pLenti4/BLOCK-iT™-DEST vector, which consisted of mixing 150 ng entry clone with 150 ng destination vector and this solution was made up to 8 µl with TE buffer, pH 8.0 (**Table 2.8**). LR Clonase™ II enzyme mix was removed from -20°C and thawed on ice, after which it was briefly vortexed twice (about 2 seconds each time). Two µl of LR Clonase™ II enzyme mix was added to the 8 µl of plasmids and mixed well by pipetting. This mixture was incubated at 25°C for 1 hour and then 1 µl of Proteinase K solution (**Table**

2.8) was added and incubated for 10 minutes at 37°C to remove protein contamination. The newly formed pLenti4/BLOCK-iT™ expression construct was then transformed into One Shot® Stbl3™ competent *E. coli* as this strain is particularly well-suited for use in cloning unstable DNA such as lentiviral DNA containing direct repeats.

2.14.4 Transforming One Shot® Stbl3™ competent *E. coli*

One vial of One Shot® Stbl3™ competent *E. coli* was thawed on ice and 3 µl of the ligation reaction from section **2.14.3** was mixed in gently before the vial was incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 45 seconds without shaking before incubating on ice for a further 2 minutes. 250 µl of pre-warmed S.O.C medium (**Appendix 1**) was added to the vial and was shaken horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator. Twenty five µl and 50 µl of the transformation mixture was plated onto LB agar plates containing 50 µg/ml ampicillin to select for cells that have incorporated the expression construct. A single colony was picked and grown in 2 ml LB broth overnight at 37°C with shaking at 225 rpm; this broth was used to inoculate 100 ml of LB broth and was grown for a further 16 hours at 37°C with shaking at 225 rpm. Plasmid DNA was harvested using a Plasmid Plus Maxi Kit (Qiagen) according to manufacturer's instructions and resuspended at 1 µg/ml in TE buffer.

2.14.5 Producing Lentivirus in 293FT cells

Lentiviral stocks were produced in HEK 293FT cells by transfecting a 10 cm cell culture dish of a confluent healthy monolayer of HEK 293FT with 9 µg of ViraPower™ Packaging Mix (**Table 2.8**) and 3 µg of pLenti4/BLOCK-iT™ expression construct maxi prep using 36 µl of Lipofectamine 2000™ transfection reagent (protocol can be found in **section 2.9**). The ViraPower™ Packaging Mix contains three packaging plasmids required for the production of the lentivirus. The map and features of these packaging plasmids can be found in **Appendix 1**. Virus-containing supernatant was collected 72 hours post-transfection and cell debris was pelleted by centrifugation at 3000 rpm for 5 minutes at +4°C. The virus was then concentrated using the MiniMate™ tangential flow system.

2.14.6 Concentration of lentiviral particles

Viral supernatants were concentrated using a tangential flow filtration system (Minimate™, Pall Corporation, Farlington, UK) and buffer exchanged with phenol-red-free DMEM. Viral supernatants from several different wells that had been transfected with ViraPower™ Packaging Mix and pLenti4/BLOCK-iT™ expression construct were pooled and concentrated from approximately 100 ml to 5 ml. The tangential flow filtration system was set up

according to manufacturer's instructions, which utilises a motor pump to keep the system at low pressure. Separate 100 kDa (10 nm pore size) filters (Minimate™ TFF capsules) were used for each viral preparation and the resultant concentrated media was then topped up to 20 ml with phenol-red-free DMEM and concentrated three times before collecting the concentrated virus. Virus was snap frozen in liquid nitrogen and stored in small aliquots of 50 – 500 µl at -80°C until required. Concentrated virus was not freeze-thawed.

2.14.7 Zeocin™ kill curve experiment

Zeocin™ is a broad spectrum antibiotic that belongs to a family of structurally related bleomycin/phleomycin-type antibiotics isolated from *Streptomyces*. Zeocin™ has a working concentration of 50 – 1000 µg/ml in mammalian cells and a kill curve experiment was carried out to determine the minimum concentration of Zeocin™ required to kill all untransduced cells. The kill curve was carried out by plating HeLa and BEAS-2B cells in a 24-well plate at approximately 25 % confluence. The cells were incubated at 37°C + 5 % CO_2 overnight to allow adherence, followed by the addition of a range of 50 – 1000 µg/ml (50 µg/ml intervals) of Zeocin™. This selective media was replenished every 3-4 days and the concentration of Zeocin™ that killed the cells within 10-14 days after addition was used for the selection of Zeocin™-resistant cells in future transduction experiments. It is important to note that Zeocin™ method of killing is different to that of other common antibiotics, so Zeocin™-sensitive cells do not round up and detach from the plate but show a vast increase in size; abnormal cell shape; presence of large empty vesicles in the cytoplasm where organelles have broken down; and breakdown of plasma and nuclear membrane. The concentration of Zeocin™ required to kill BEAS-2B and HeLa cells was 150 µg/ml and 250 µg/ml, respectively.

2.14.8 Determining lentiviral titre

Concentration of infectious viral particles was determined by induction of antibiotic resistance in HeLa cells. The cells were grown to 50 % confluence in a 6-well plate and on the day of transfection (day 1), lentiviral stocks were thawed and 10-fold serial dilutions ranging from 10^{-2} to 10^{-6} were made in complete media up to the volume of 1 ml. Spent cell culture medium was removed from cells and each dilution of virus, along with 10 µg/ml Polybrene® (**Table 2.8**) to enhance transduction of lentivirus into mammalian cells, was cultured at 37°C + 5 % CO_2 overnight. An untransfected control that contained no virus was also included. The following day (day 2), the media containing virus was removed and was replaced with 2 ml of complete media and cultured overnight at 37°C + 5 % CO_2. On day 3, each well of cells was trypsinised and replaced in a separate 10 cm plate containing

complete media with the appropriate amount of Zeocin™. This selective medium was changed every 2-3 days for 10-12 days until there were no live cells in the untransfected plate. The plates were then washed twice in 1X PBS and 5 ml of crystal violet solution (**Table 2.8**) was added and incubated for 10 minutes at room temperature. The crystal violet solution was then removed and cells were washed twice in 1X PBS. Blue stained colonies were counted in appropriate wells (some wells will contain too many colonies and therefore the number will be undeterminable) and averaged to determine the titre of the virus. The titre of the lentivirus carrying the Pellino1 shRNA was 2.83 x 10^7 transducing units (TU)/ml and the lentivirus carrying the MyD88 shRNA was 1.06 x 10^7.

Reagent	**Composition**
TE buffer	10 mM Tris-HCl, pH 8.0 1 mM EDTA
LR Clonase™ II enzyme mix	Proprietary
Proteinase K Solution	2 µg/µl in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50 % Glycerol
Virapower™ Packaging Mix	A combination of pLP1, pLP2 and pLP/VSVG plasmids, lyophilized in TE buffer, pH 8.0
Polybrene®	hexadimethrine bromide
Crystal violet solution	1 % crystal violet solution in 10 % ethanol

Table 2.8: Composition of reagents required for the manufacturing of Lentivirus

The table outlines the composition of the reagents supplied in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System used in the manufacture of a lentiviral vector carrying the pLenti4/BLOCK-iT™-DEST vector encoding a shRNA that is used to target specific genes for silencing via the RNAi pathway.

2.14.9 Transient transduction of shRNA using lentiviral vectors in HeLa and BEAS-2B cells

HeLa and BEAS-2B cells were seeded into 24-well plates and grown to approximately 80 % confluency. On the day of transduction, the cells were washed in 1X PBS before the addition of 500 µl of complete fresh media. Amount of virus to add was calculated using the equation:

$$Amount\ of\ virus\ (ml) = \frac{number\ of\ cells\ per\ well\ x\ MOI\ desired}{Titre}$$

The correct amount of virus for a M.O.I of 1 or 5 was added to the appropriate wells along with 10 µg/ml of Polybrene®. These cells were cultured at 37°C + 5 % CO_2 for 24 hours before the media was replaced with 500 µl of complete fresh media. These cells were cultured for a further 24 hours at 37°C + 5 % CO_2 before RNA was harvested using Tri-reagent.

2.14.10 Single cell cloning

Lentivirus carrying shRNA targeting Pellino1 or MyD88 was used to create stable gene knockdowns by transducing the cells with the lentivirus and selecting for cells that had integrated the expression construct into the chromosome. This was carried out by transducing HeLa and BEAS-2B cells with lentiviral particles carrying either MyD88 or Pellino1 shRNA at a M.O.I of 5 (as outlined in **section 2.14.9**) and 48 hours post-transduction, cells were treated with the appropriate concentration of Zeocin™ (as outlined in **section 2.14.7**). Selective media was replenished every 2-3 days to select for cells that had integrated the expression construct in their chromosomes and these cells were grown to confluence. Cells were then trypsinised and counted, then diluted to the concentration of 100-, 50- and 10-cells/ml. One hundred µl of each dilution was plated into each well of a separate 96-well plate to give plates containing 10-, 5- and 1- cell/well, respectively. These cells were then cultured at 37°C + 5 % CO_2 for 1 week. After this time, plates were scanned by light microscopy for wells that contained only 1 colony of cells. Wells containing more than 1 colony were disregarded as these would not contain a homogeneous population of cells and therefore levels of expression of the shRNA would not be uniform due to insertion of the expression construct in different loci in the chromosome. Up to 16 wells from each condition (HeLa Pellino1 knockdown; BEAS-2B Pellino1 knockdown; HeLa MyD88 knockdown; and BEAS-2B MyD88 knockdown) were picked and passaged through 24- and 6-well plates up to T25 tissue culture flasks. Zeocin™ antibiotic selective pressure was present throughout the culturing process to ensure

complete selection of transduced cells. Target gene silencing was visualised using RT-PCR and the clones that exhibited the most prominent gene knockdown at the mRNA level were taken forward for further quantification of knockdown and functional studies.

2.15 Specific methods for the creation of Pellino1 stable BEAS-2B cell-lines using the Sigma MISSION™ system

The Sigma MISSION™ system provides 4 ready-made lentiviral vectors each containing a different shRNA targeting Pellino1, the sequences of which can be found in **Appendix 1**. The efficiency of each virus to lead to Pellino1 knockdown was tested by transiently transducing BEAS-2B cells with each virus. Cells were transduced with a M.O.I of 1 as outlined in **section 2.14.9.**

The virus that led to the greatest knockdown of Pellino1 with transient transduction was then used to generate a stable Pellino1 knockdown cell-line. This was achieved by transducing BEAS-2B cells at an M.O.I of 1 and 3 and 24 hours post-transduction, 0.3 µg/ml puromycin was added to select for cells that had incorporated the shRNA-containing construct into the chromosome. The appropriate amount of puromycin to select for transduced cells was determined by a kill curve experiment (see **Section 2.14.7**). Selective media was replenished every 2-3 days and transduced cells were single-cell cloned (see **Section 2.14.10**). Ten clones from each M.O.I were analysed for target gene knockdown at the mRNA level

2.16 Specific methods for the creation of Tet repressor expressing BEAS-2B cell-line

A Tet Repressor-expressing BEAS-2B cell-line was to be generated to allow for inducible shRNA production leading to Pellino1 knockdown.

2.16.1 How Tetracycline Regulation works

The BLOCK-iT™ Inducible H1 RNAi system uses regulatory elements from the *E. coli* Tn10-encoded tetracycline (Tet) resistance operon to allow tetracycline-regulated expression of the shRNA of interest. As previously described, the shRNA is under the control of the human H1 promoter, which has been modified to incorporate 2 copies of the *tet* operator 2 ($TetO_2$) sequence (H1/TO promoter). Each copy of $TetO_2$ allows binding of 2 Tet repressor proteins and the binding of 4 Tet repressor proteins to each H1/TO promoter leads to repression of the shRNA of interest. The antibiotic tetracycline binds with high affinity to Tet repressor proteins and causes a conformational change that renders them unable to

bind to $TetO_2$ sequences, thus allowing transcription of the shRNA leading to gene silencing. Tetracycline regulation is outlined in **Figure 2.1**.

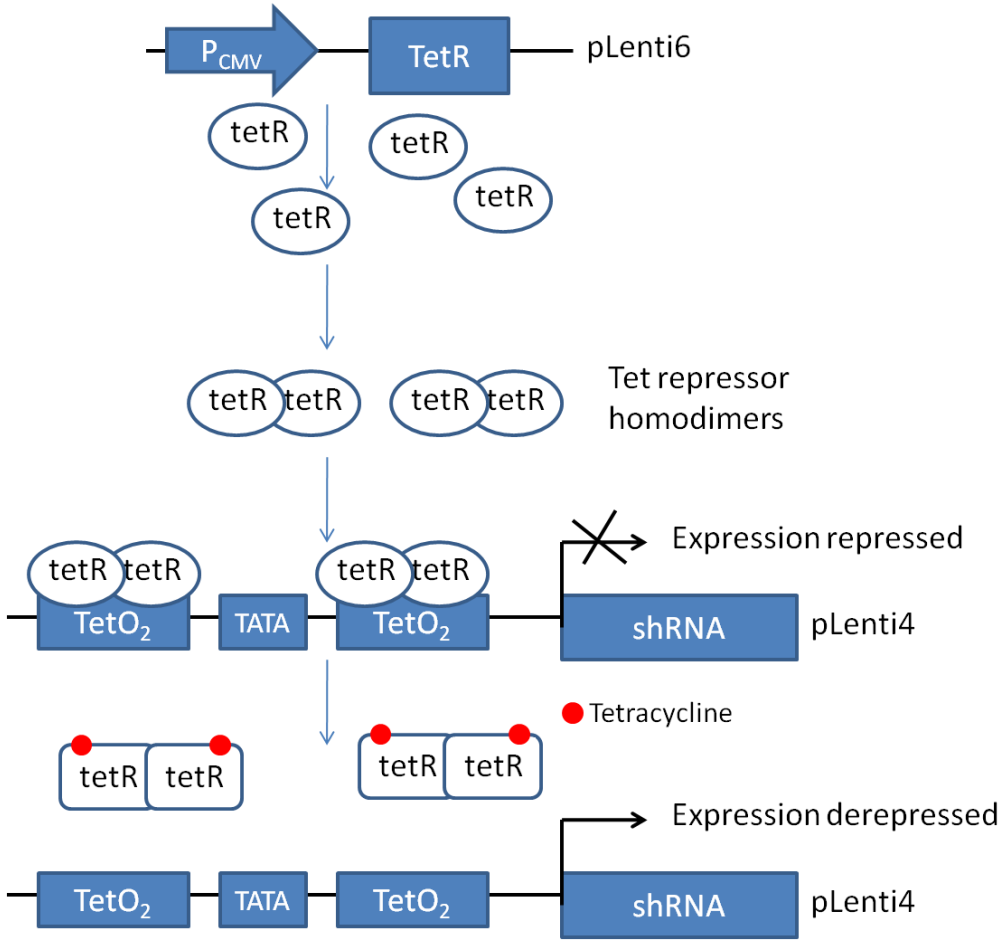


Figure 2.1: How Tetracycline regulation works

The BLOCK-iT™ Inducible H1 RNAi system allows for tetracycline-regulated shRNA expression by transcribing the Tet repressor protein that is under the CMV promoter in the pLenti6/TR plasmid. The Tet repressor protein forms homodimers that can bind to $TetO_2$ sequences in the H1/TO promoter in the pLenti4-expression construct, leading to repression of the shRNA of interest. Upon addition, tetracycline binds to and changes the conformation of the Tet repressor, which renders it unable to bind to $TetO_2$ sequences, thus allowing transcription of the shRNA and consequential gene knockdown.

2.16.2 Creating a Tet repressor expressing host cell line

This was achieved by the packaging of the pLenti6/TR plasmid (plasmid map and features can be found in **Appendix 1**) encoding the Tet repressor protein into a lentivirus as outlined in **sections 2.14.4 – 2.14.8**. The resultant lentivirus was used to transiently transduce BEAS-2B cells at a M.O.I of 10 to ensure that it is capable of delivering the pLenti6/TR plasmid into the cells (as outlined in **sections 2.14.9**).

This lentivirus was then used to generate a BEAS-2B cell-line that stably produces the Tet repressor protein. After transduction with the lentivirus carrying the pLenti6 plasmid, BEAS-2B cells that had incorporated the construct into the chromosome were selected for by adding the 5 μg/ml of blasticidin, which was determined by performing a kill curve experiment (**section 2.14.7**). Untransduced cells were selected out by replacing the selective media every 3-4 days for 14 days.

2.17 Statistical analysis

Statistical analyses were performed using Prism Software (Version 5.0 GraphPad, San Diego, CA). Multiple data sets were analysed using one-way or two-way ANOVA with Bonferroni selected pairs post-tests and single data comparisons used either a paired or unpaired t-tests dependent on the data sets. Significance is represented by *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

3 Chapter 3 – Results. Expression and regulation of the Pellino family.

3.1 Aims

The family of mammalian Pellino proteins is made up of Pellino1, Pellino2, and the splice variants Pellino3a and Pellino3b. Expression of the murine orthologues of Pellino1 and Pellino2 has previously been shown to be tissue specific at the transcriptional level (Yu, Kwon et al. 2002; Jiang, Johnson et al. 2003). Human Pellino3 mRNA was found to be expressed at high levels in brain, heart and testis and at lower levels in kidney, liver, lung, placenta, small intestine, spleen and stomach. Only very low levels of human Pellino3 could be detected in colon and muscle (Jensen and Whitehead 2003). Pellino expression has also been shown to be modulated in response to proinflammatory stimuli such as LPS or poly(I:C) (Weighardt, Jusek et al. 2004; Smith, Liu et al. 2011).

The aims of the data presented in this chapter were to undertake pilot experiments to gain some insight into patterns of Pellino expression and potential regulation in response to proinflammatory agonists at the mRNA level (using RT-PCR and qPCR) in primary human lung airway epithelium, primary human leukocytes, and the human cell-lines BEAS-2B, HeLa and HEK 293FT cells, prior to proceeding to exploration of functional roles for Pellinos. The two commercially available antibodies raised against Pellino1 were tested in BEAS-2B cells.

3.2 Expression of Pellino family in tissue cells

Pellino expression in the human cell-lines HeLa, HEK and BEAS-2B were compared to the expression pattern of Primary Human Bronchial Epithelial cells (PBECs) using RT-PCR. The cervical cancer cell-line, HeLa, and human embryonic kidney cell line, HEK 293FT, cells express all of the Pellino family (**Figure 3.1**). Both the immortalised lung airway epithelial cell-line, BEAS-2B, and PBECs express Pellino1, Pellino3a and Pellino3b, however Pellino2 transcripts were unable to be detected after 30 PCR cycles (**Figure 3.1**).

Pellino1 expression at the protein level was explored using two commercially available antibodies (obtained from Abcam and Santa Cruz, raised to two different epitopes). **Figure 3.2** shows the immunogenic band detected at the expected size (46 kDa) was visualised in different compartments of membrane and cytosolic fractions, suggesting that one or both of these antibodies detect a non-specific cellular product. These data, along with the lack of protein knockdown in lysates that show a significant reduction of Pellino1 mRNA (data not

shown), led us to believe that these antibodies were not reliable and therefore worth studying. Pellino1 expression at the protein level was not pursued any further.

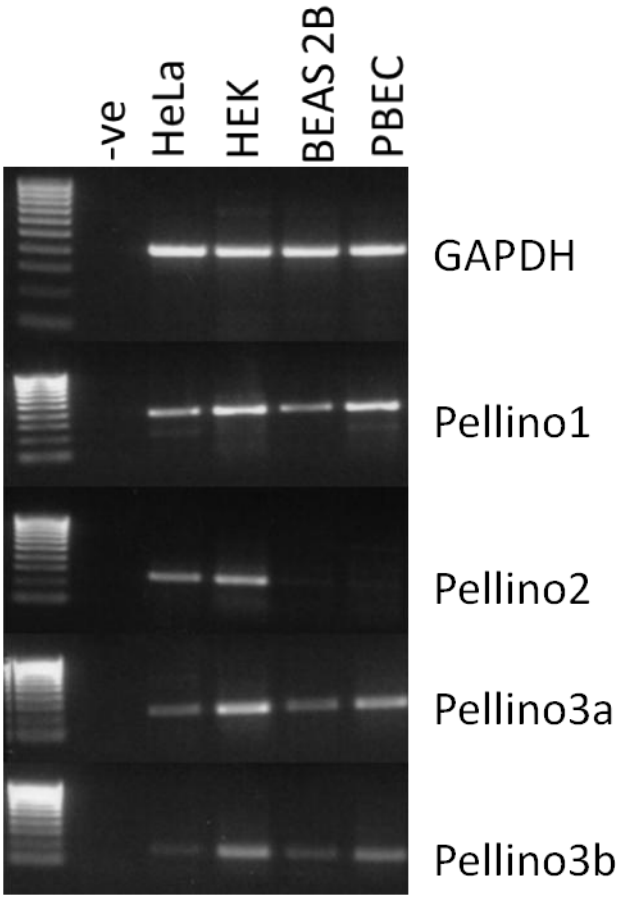


Figure 3.1: Pellino family mRNA expression in tissue cells

Pellino mRNA expression was explored using RT-PCR. HeLa, HEK 293FT (HEK), BEAS-2B and PBEC cDNA was used to amplify either Pellino1, Pellino2, Pellino3a, Pellino3b or the loading control GAPDH using specific primers. DNA bands after 30 PCR cycles were separated using agarose gel electrophoresis and visualised using a UV-transilluminator. A no template negative control was also included (-ve). HeLa and HEK cells expressed all of the Pellino family, whereas Pellino2 transcripts could not be detected in BEAS-2B or PBECs. Representative gels from 2 independent experiments.

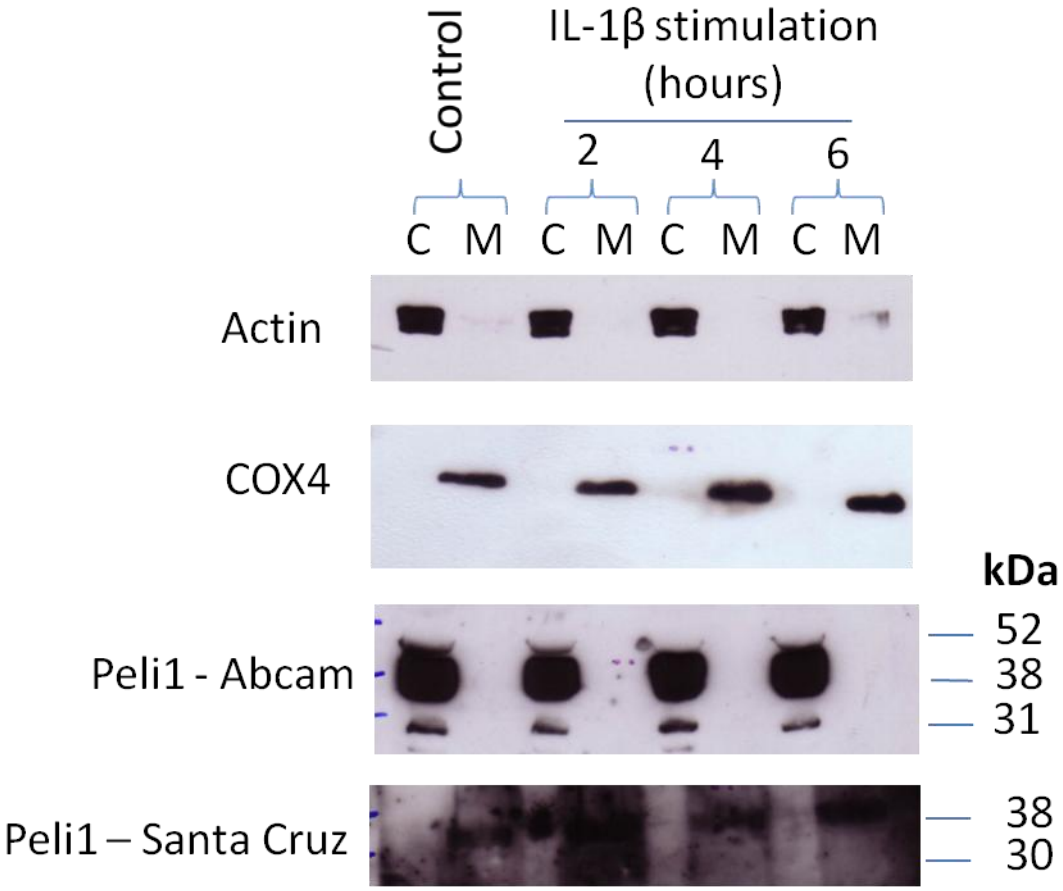


Figure 3.2: Testing Pellino1 antibodies using cytosolic and membrane fractions in BEAS 2B cells

Cytosolic and membrane fractions were extracted from BEAS-2B cells that had been stimulated with 10 ng/ml IL-1β for 2-6 hours. These lysates were subjected to western blotting and the cytoplasmic control (actin) and the membranous control (COX4 – mitochondrial protein) were only visualised in the expected compartments, suggesting the fractions had been separated sufficiently. The Pellino1 antibody from Abcam bound to its epitope in the cytosolic fractions and the Pellino1 antibody from Santa Cruz bound to its epitope in the membranous fraction. These data suggest that these antibodies do not bind to the same protein.

3.3 Expression and regulation of Pellino family in monocytes

Monocytes are mononuclear cells that play a key role in the innate immune response where they are involved in phagocytosing foreign material, antigen presentation and the production of cytokines. As key inflammatory cells, the regulation of inflammatory signalling is tightly controlled. Since Pellino expression has previously been shown to be regulated at the transcriptional level in response to TLR agonists in murine cells (Weighardt, Jusek et al. 2004; Smith, Liu et al. 2011), the mRNA expression pattern of the Pellino family in IL-1β or LPS-stimulated primary human monocytes was analysed using RT-PCR. Primary human monocytes express all of the Pellino family and this expression pattern does not change at the transcriptional level after 1 hour of IL-1β or LPS treatment (**Figure 3.3**).

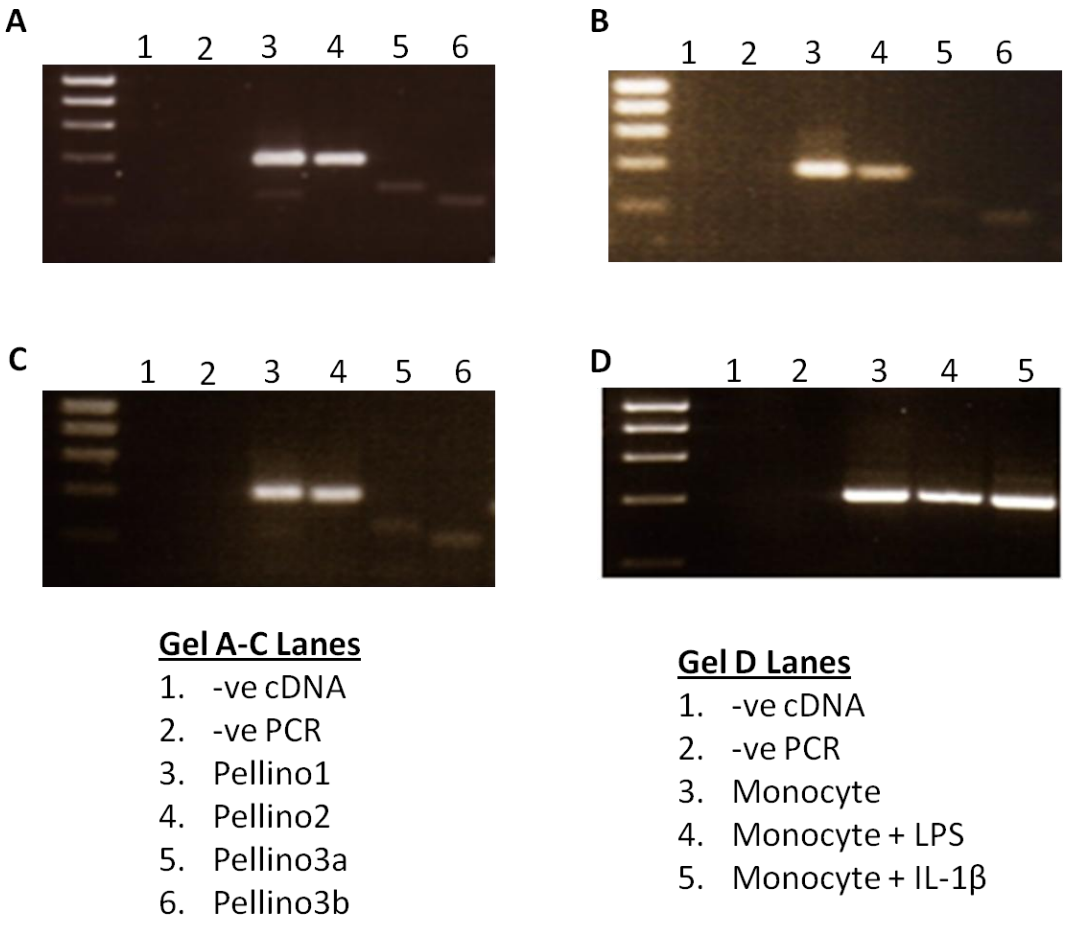


Figure 3.3: Monocyte Pellino expression and regulation

Pellino mRNA expression and regulation in monocytes was explored using RT-PCR. RNA from monocytes that were either unstimulated (**A**), or had been treated with 10 ng/ml LPS (**B**) or 10 ng/ml IL-1β (**C**) for 1 hour was extracted using Tri-reagent and cDNA was used to amplify either Pellino1, Pellino2, Pellino3a, Pellino3b or the loading control GAPDH using specific primers. DNA bands after 30 PCR cycles were separated using agarose gel electrophoresis and visualised using a UV-transilluminator. A no template negative control was also included in both the reverse transcriptase (-ve cDNA) and PCR reactions (-ve PCR). Unstimulated monocytes expressed all of the Pellino family (**A**) and this expression pattern did not significantly change at the transcriptional level in response to LPS (**B**) or IL-1β (**C**) treatment for 1 hour. GAPDH loading controls are comparable between treatments (**D**). Representative gels from 2 independent experiments.

3.4 Expression and regulation of Pellino family in neutrophils

Neutrophils are polymorphonuclear granulocytes, whose main function is to engulf and degrade pathogens. Up to two thirds of circulating blood leukocytes are comprised of neutrophils and these cells play an important role in innate immune defences. Either unstimulated neutrophils or neutrophils that had been treated with IL-1β, LPS or TNFα for 1 or 3 hours were used to analyse Pellino mRNA expression by RT-PCR. Unstimulated neutrophils expressed Pellino1 (**Figure 3.4b**), Pellino2 (**Figure 3.4c**) and low levels of Pellino3b (**Figure 3.4e**), however Pellino3a transcripts were below the level of detection after 30 PCR cycles (**Figure 3.4d**). Stimulation of neutrophils with IL-1β, LPS or TNFα for 1 or 3 hours had no effect on Pellino mRNA expression (**Figure 3.4**).

RT-PCR is a relatively insensitive method of detecting changes in mRNA levels as it is not quantifiable and therefore can only be used to visualise on/off transcriptional changes. Quantitative PCR is capable of detecting subtle changes in transcript levels in response to stimulus and this was used to measure Pellino1 transcriptional changes in response to IL-1β, LPS or TNFα for 1 or 3 hours in neutrophils. Neutrophils were found to have slightly increased Pellino1 transcript levels in cells that had been treated with LPS, TNFα or IL-1β (to a lesser extent) for 3 hours (**Figure 3.5**).

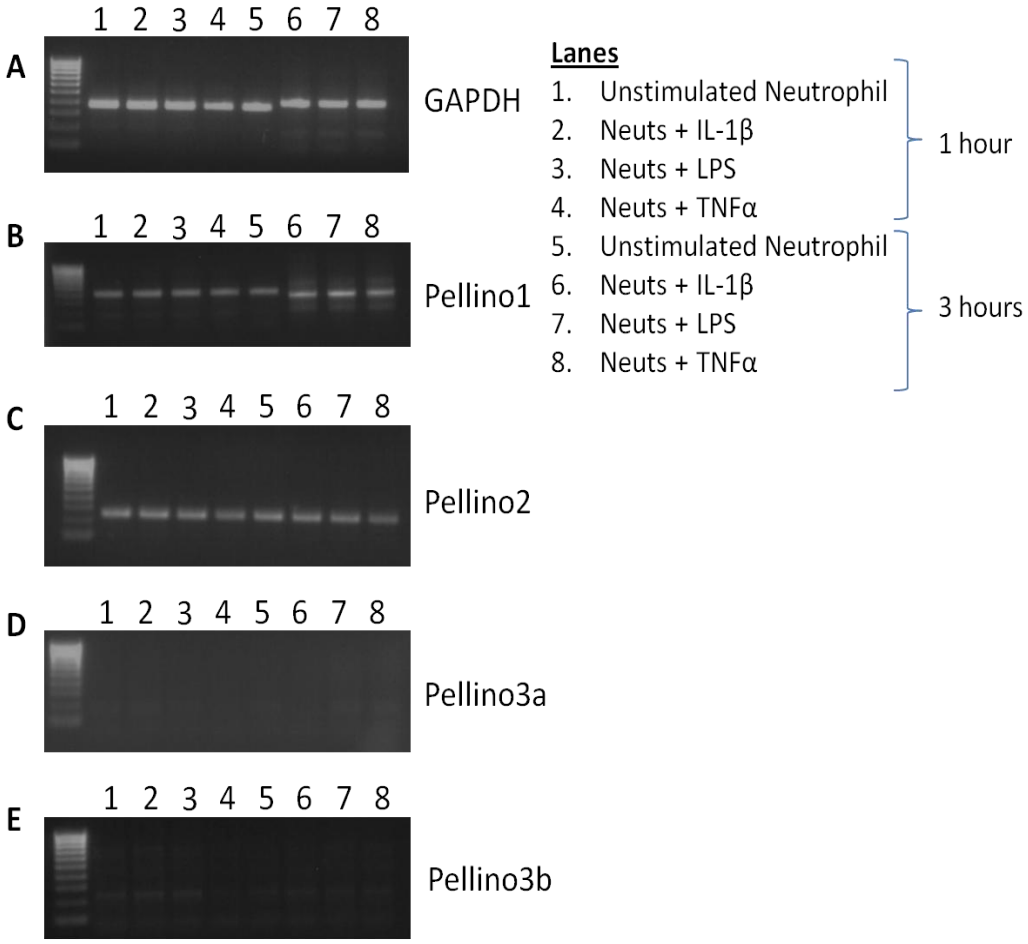


Figure 3.4: Expression and regulation of Pellino family in neutrophils

Pellino mRNA expression and regulation in neutrophils was explored using RT-PCR. RNA from neutrophils that were either unstimulated, or had been treated with 10 ng/ml LPS, IL-1β or TNFα for 1 or 3 hours was extracted using Tri-reagent and cDNA was used to amplify either Pellino1, Pellino2, Pellino3a, Pellino3b or the loading control GAPDH using specific primers. DNA bands after 30 PCR cycles were separated using agarose gel electrophoresis and visualised using a UV-transilluminator. Unstimulated neutrophils expressed Pellino1 (**B**), Pellino2 (**C**) and low levels Pellino3b (**E**) transcripts, however Pellino3a transcripts were unable to be detected after 30 PCR cycles (**D**). This expression pattern did not change at the transcriptional level in response to IL-1β, LPS or TNFα treatment for 1 or 3 hours. GAPDH loading controls are comparable between treatments (**A**). Representative gels from 2 independent experiments.

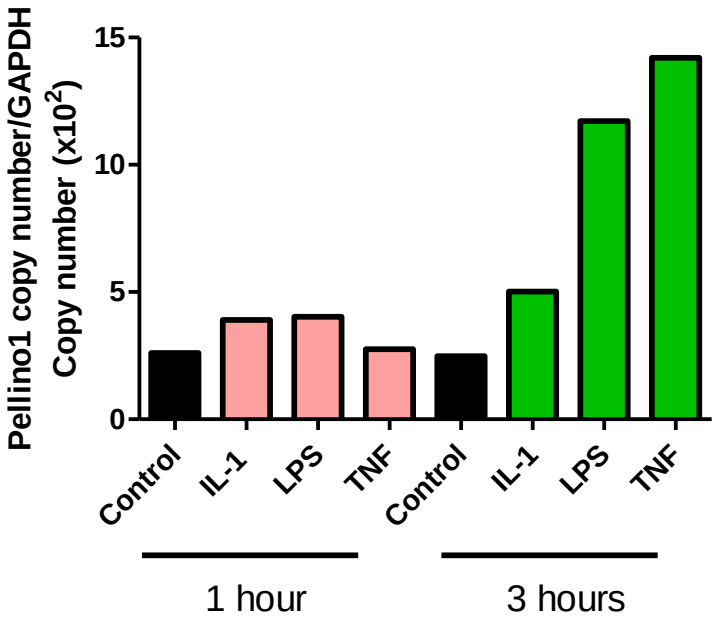


Figure 3.5: Pellino1 regulation in human primary neutrophils

Pellino1 mRNA expression and regulation in neutrophils was explored using qPCR. RNA from neutrophils that were either unstimulated, or had been treated with 10 ng/ml LPS, IL-1β or TNFα for 1 or 3 hours was extracted using Tri-reagent. Pellino1 or GAPDH (control) specific qPCR primer-probes were used to amplify target genes and fluorescence was measured using the ABI 7900HT Fast Real-Time PCR System. Unstimulated neutrophils expressed Pellino1 and this expression level did not change after 10 ng/ml IL-1β, LPS or TNFα stimulation for 1 hour or no stimulus for 3 hours. Pellino1 transcripts were increased in neutrophils after 3 hours of LPS, TNFα and IL-1β (to a lesser extent) stimulation. Data are expressed as a ratio of Pellino1 copy number to GAPDH control copy number and are from a single experiment.

3.5 Regulation of Pellinos in BEAS-2B cells

BEAS-2B cells are a lung airway epithelial cell-line that was also used in later experiments to investigate the function of Pellino1 in lung airway epithelial inflammatory responses (**see Chapter 4+5**). Previous data suggested that BEAS-2B cells do not express Pellino2 at the transcriptional level (**Figure 3.1**). To investigate whether this expression pattern changes upon stimulation with IL-1β, the viral mimic poly(I:C) or TNFα for 1 or 3 hours, mRNA from these cells was extracted using Tri-reagent and Pellino mRNA expression was measured using RT-PCR. Unstimulated BEAS-2B expressed Pellino1 (**Figure 3.6b**) and low levels of Pellino3a (**Figure 3.6d**) transcripts, however Pellino2 transcripts were unable to be detected after 30 PCR cycles (**Figure 3.6c**). This expression pattern did not change at the transcriptional level in response to IL-1β, poly(I:C) or TNFα treatment for 1 or 3 hours (**Figure 3.6b-d**). Pellino3b transcripts were expressed in unstimulated BEAS-2B cells and showed a slight reduction in transcript levels in response to IL-1β, LPS and TNFα for 1 and 3 hours (**Figure 3.6e**).

Quantitative PCR is capable of detecting subtle changes in transcript levels in response to stimulus and previous data suggested that Pellino1 transcripts are upregulated after 3 hours of IL-1β, LPS and TNFα stimulation in human neutrophils (**Figure 3.5**). Quantitative PCR was used to measure Pellino1 transcriptional changes in response to IL-1β, poly(I:C) or TNFα for 1 or 3 hours in BEAS-2B. BEAS-2B express Pellino1 and exhibit a modest reduction in Pellino1 transcripts in response to IL-1β, poly(I:C) or TNFα treatment for 1 and 3 hours (**Figure 3.7**).

The human pathogen rhinovirus1B (RV1B) infects the upper and lower respiratory tract and has been shown to be a major cause of asthma exacerbations (Atmar, Guy et al. 1998; Grissell, Powell et al. 2005). BEAS-2B cells were infected with RV1B for 1 hour and Pellino1 mRNA levels were measured using qPCR 24 hours post-infection. Pellino1 mRNA was slightly reduced in response to rhinoviral infection (**Figure 3.8**).

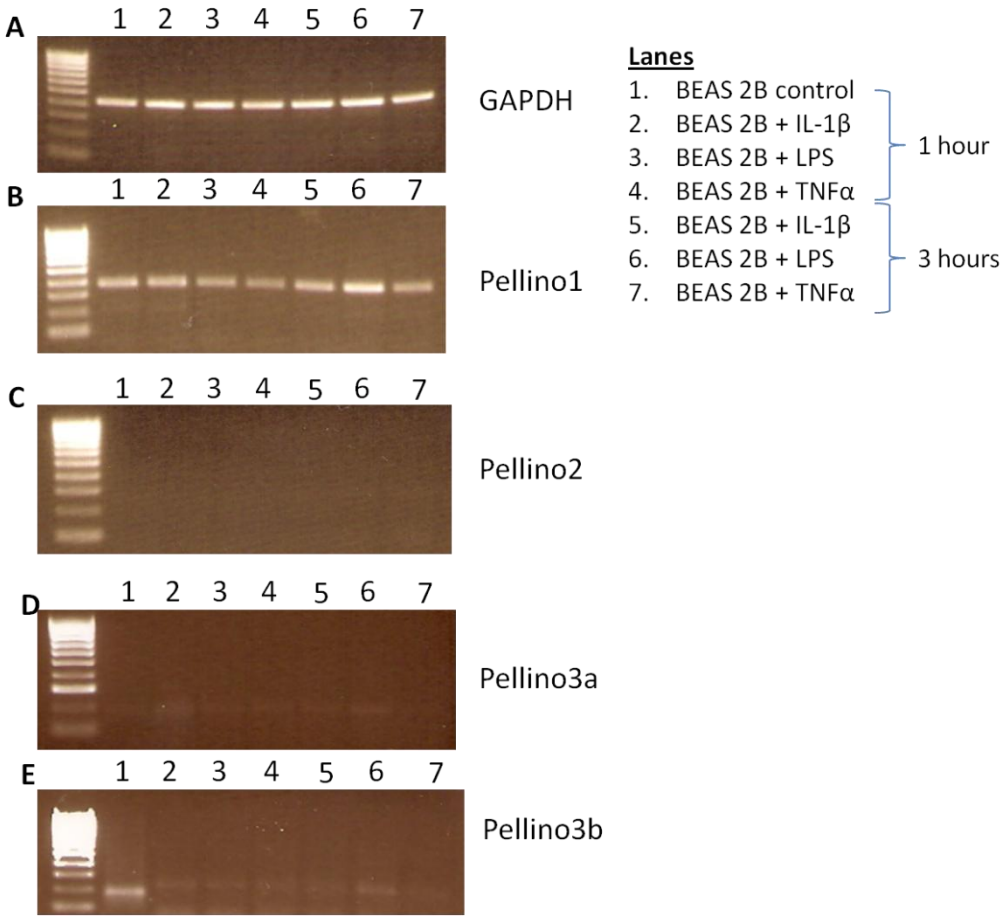


Figure 3.6: Regulation of Pellinos in BEAS-2B cells

Pellino mRNA expression and regulation in the lung airway epithelial cell-line BEAS-2B was explored using RT-PCR. RNA from BEAS-2B that were either unstimulated, or had been treated with 10 ng/ml IL-1β, 10 µg/ml poly(I:C) or 10 ng/ml TNFα for 1 or 3 hours was extracted using Tri-reagent and cDNA was used to amplify either Pellino1, Pellino2, Pellino3a, Pellino3b or the loading control GAPDH using specific primers. DNA bands after 30 PCR cycles were separated using agarose gel electrophoresis and visualised using a UV-transilluminator. Unstimulated BEAS-2B expressed Pellino1 (**B**) and low levels of Pellino3a (**D**) and Pellino3b (**E**) transcripts, however Pellino2 transcripts were unable to be detected after 30 PCR cycles (**C**). This expression pattern did not change at the transcriptional level in response to IL-1β, poly(I:C) or TNFα treatment for 1 or 3 hours. GAPDH loading controls are comparable between treatments (**A**). Representative gels from 2 independent experiments.

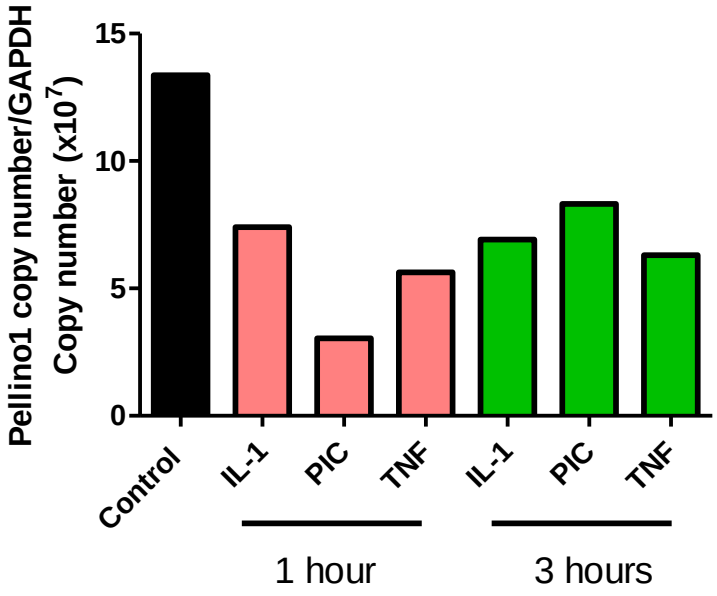


Figure 3.7: Pellino1 regulation in response to IL-1β, poly(I:C) and TNFα in BEAS-2B cells

Pellino1 mRNA expression and regulation in BEAS-2B cells was explored using qPCR. RNA from BEAS-2B cells that were either unstimulated (control), or had been treated with 10 ng/ml IL-1β, 10 µg/ml poly(I:C) or 10 ng/ml TNFα for 1 or 3 hours was extracted using Tri-reagent. Pellino1 or GAPDH (control) specific qPCR primer-probes were used to amplify target genes and fluorescence was measured using the ABI 7900HT Fast Real-Time PCR System. Unstimulated BEAS-2B expressed Pellino1 and this expression was modestly decreased after stimulation with IL-1β, poly(I:C) or TNFα. Data are expressed as a ratio of Pellino1 copy number to GAPDH control copy number and are from a single experiment.

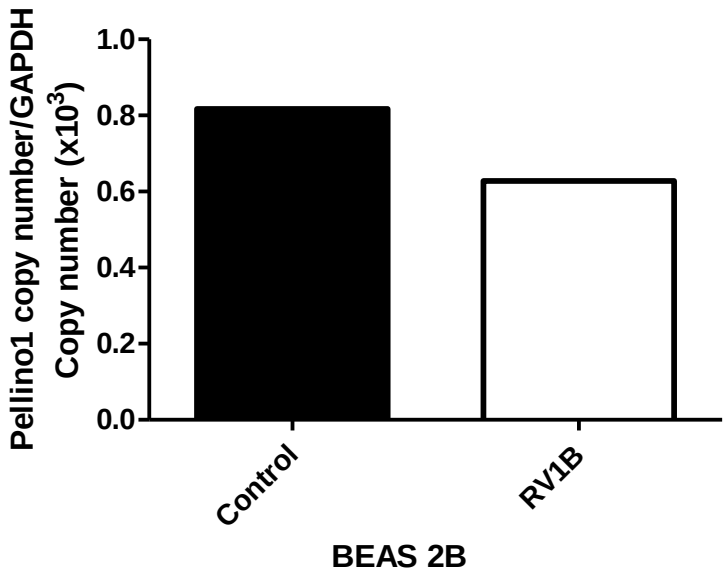


Figure 3.8: Pellino1 regulation in response to rhinovirus1B infection in BEAS-2B cells

Pellino1 mRNA regulation in response to rhinovirus1B (RV1B) infection in BEAS-2B cells was explored using qPCR. RNA from BEAS-2B cells that had been infected with a $TCID_{50}$ of 2 x 10^7 for 1 hour was extracted using Tri-reagent. Pellino1 or GAPDH (control) specific qPCR primer-probes were used to amplify target genes and fluorescence was measured using the ABI 7900HT Fast Real-Time PCR System. BEAS-2B cells infected with RV1B showed a modest reduction Pellino1 mRNA levels compared to unstimulated BEAS-2B cells. Data are expressed as a ratio of Pellino1 copy number to GAPDH control copy number and are from a single experiment.

3.6 Regulation of Pellinos in primary bronchial epithelial cells

Commercially sourced primary human bronchial epithelial cells (PBECs) that were originally isolated from healthy volunteers via bronchoscopy, were used later in this thesis to investigate the function of Pellino1 in lung airway epithelial inflammatory responses (**See Chapter 5**). Previous data suggested that PBECs exhibit the same expression pattern of the Pellino family as BEAS-2B cells, in that they do not express Pellino2 at the transcriptional level (**Figure 3.1**). To investigate whether this expression pattern changes upon stimulation with IL-1β, LPS or TNFα for 1 or 3 hours, mRNA from these cells was extracted using Tri-reagent and Pellino mRNA expression was measured using RT-PCR. Unstimulated PBECs expressed Pellino1 (**Figure 3.9b**) and low levels of Pellino3a (**Figure 3.9d**) and Pellino3b (**Figure 3.9e**) transcripts, however Pellino2 transcripts were not detected after 30 PCR cycles (**Figure 3.9c**). Pellino1 mRNA expression increased after stimulation with IL-1β or TNFα after 1 and 3 hours, however LPS had no effect on Pellino1 expression (**Figure 3.9b**). Pellino2, Pellino3a and Pellino3b expression pattern did not change at the transcriptional level in response to IL-1β, LPS or TNFα treatment for 1 or 3 hours (**Figure 3.9c-e**).

Quantitative PCR was used to measure changes in Pellino1 transcripts in response to 1 or 3 hours of IL-1β or TNFα stimulation in PBECs. As suggested by the RT-PCR data, PBECs express Pellino1 and this expression is increased in response to IL-1β or TNFα treatment for 1 or 3 hours, however LPS has no effect on Pellino1 levels (**Figure 3.10**).

Previous data has shown that Pellino1 mRNA levels are increased after stimulation with the TLR3 agonist and viral mimic poly(I:C) for 3 hours in human neutrophils (**Figure 3.4**). Quantitative PCR was used to measure the effect of poly(I:C) stimulation on Pellino1 mRNA levels in PBECs and Pellino1 was upregulated after 3 hours of poly(I:C) stimulation in PBECs (**Figure 3.11**).

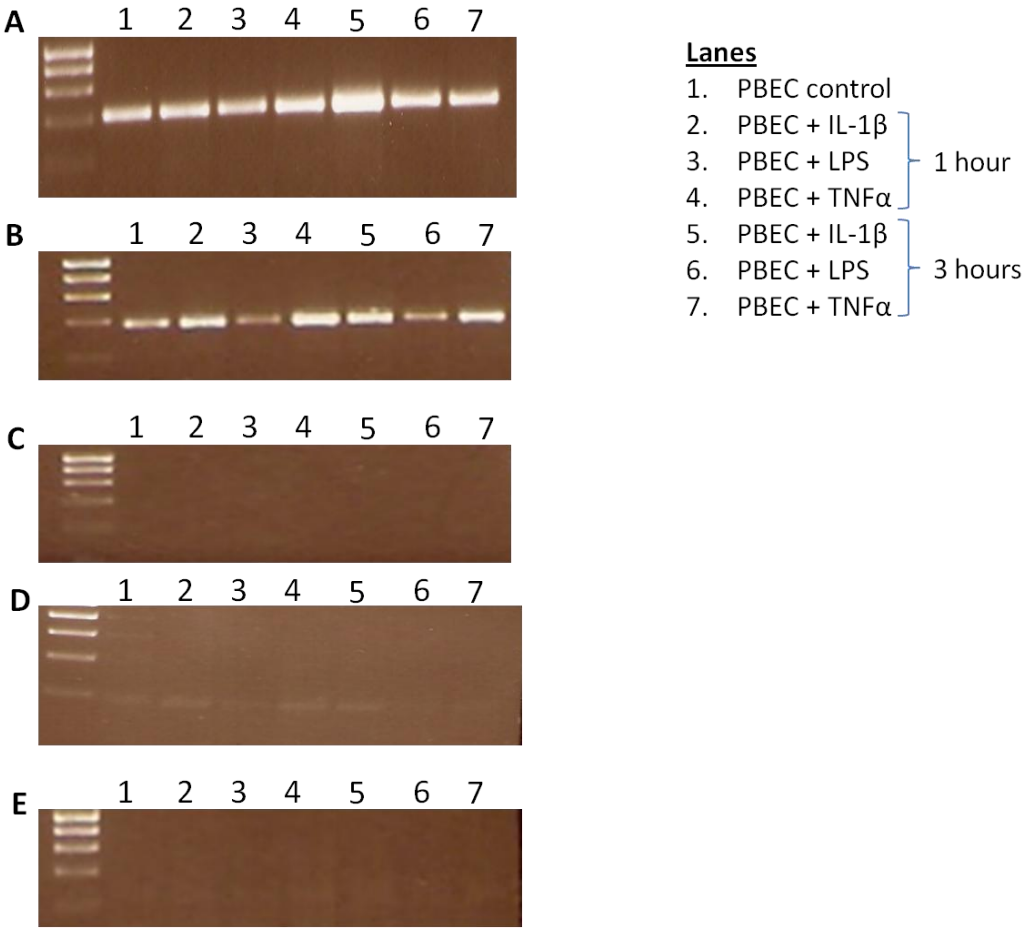


Figure 3.9: Regulation of Pellinos in primary bronchial epithelial cells

Pellino mRNA expression and regulation in PBECs was explored using RT-PCR. RNA from PBECs that were either unstimulated, or had been treated with 10 ng/ml IL-1β, 10 µg/ml poly(I:C) or 10 ng/ml TNFα for 1 or 3 hours was extracted using Tri-reagent and cDNA was used to amplify either Pellino1, Pellino2, Pellino3a, Pellino3b or the loading control GAPDH using specific primers. DNA bands after 30 PCR cycles were separated using agarose gel electrophoresis and visualised using a UV-transilluminator. Unstimulated PBECs expressed Pellino1 (**B**) and low levels of Pellino3a (**D**) and Pellino3b (**E**) transcripts, however Pellino2 transcripts were unable to be detected after 30 PCR cycles (**C**). The expression pattern was investigated at the transcriptional level in response to IL-1β, poly(I:C) or TNFα treatment for 1 or 3 hours. GAPDH loading controls are comparable between treatments (**A**). Gels are from a single experiment.

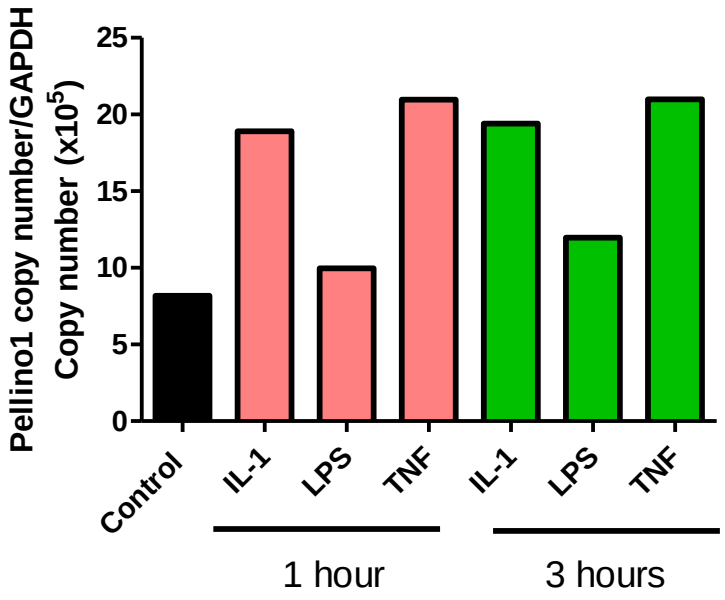


Figure 3.10: Regulation of Pellino1 in response to IL-1β, LPS and TNFα in primary bronchial epithelial cells

Pellino1 mRNA expression and regulation in PBECs was explored using qPCR. RNA from PBECs that were either unstimulated (control), or had been treated with 10 ng/ml IL-1β, LPS, or TNFα for 1 or 3 hours was extracted using Tri-reagent. Pellino1 or GAPDH (control) specific qPCR primer-probes were used to amplify target genes and fluorescence was measured using the ABI 7900HT Fast Real-Time PCR System. Unstimulated PBECs expressed Pellino1 and this expression was increased after stimulation with IL-1β or TNFα after 1 and 3 hours. Stimulation of PBECs with LPS had no effect of Pellino1 mRNA levels. Data are expressed as a ratio of Pellino1 copy number to GAPDH control copy number and are from a single experiment.

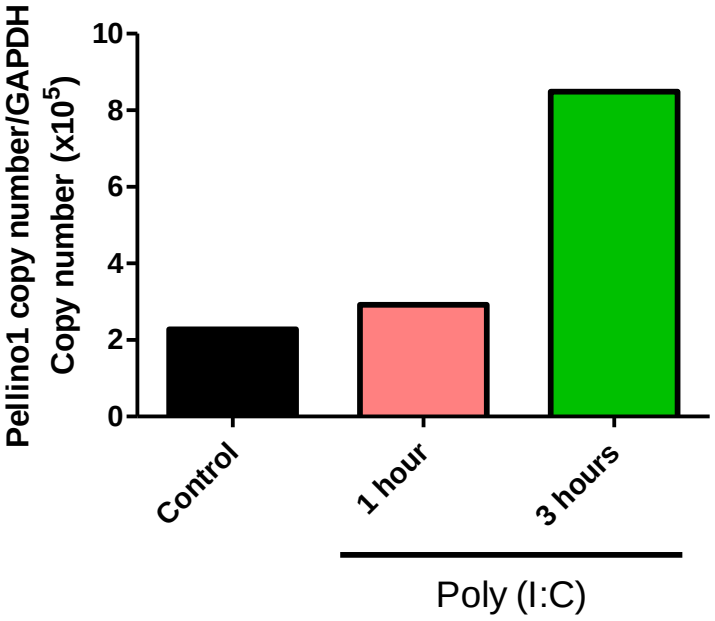


Figure 3.11: Regulation of Pellino1 in response to poly(I:C) in primary bronchial epithelial cells

Pellino1 mRNA expression and regulation in response to poly(I:C) in PBECs was explored using qPCR. RNA from PBECs that were either unstimulated (control), or had been treated with 10 µg/ml poly(I:C) for 1 or 3 hours was extracted using Tri-reagent. Pellino1 or GAPDH (control) specific qPCR primer-probes were used to amplify target genes and fluorescence was measured using the ABI 7900HT Fast Real-Time PCR System. Unstimulated PBECs expressed Pellino1 and this expression was increased after stimulation with poly(I:C) for 3 hours. Data are expressed as a ratio of Pellino1 copy number to GAPDH control copy number and are representative of 1 independent experiment.

3.7 Summary of Pellino regulation

The aim of this chapter was to develop pilot data to investigate the expression and regulation of the Pellino family in response to proinflammatory agonists in human cells. Expression and regulation was investigated at the mRNA level (using RT-PCR and qPCR) as we, and others, found that the two commercially available antibodies were not a reliable tool (Smith, Liu et al. 2011).

I found that the human cell-lines HeLa and HEK 293FT cells express all of the Pellino family, however Pellino2 transcripts in human airway cells BEAS-2B cell-line and PBECs were below the level of detection after 30 PCR cycles. Human peripheral blood monocytes express all of the Pellino family, however Pellino3a transcripts in human peripheral blood neutrophils were under the level of detection after 30 PCR cycles. RT-PCR data suggests that there are no significant changes in Pellino transcripts in primary human monocytes or neutrophils in response to IL-1β, LPS or TNFα (neutrophils only), however preliminary data from neutrophils using qPCR shows that there may be a modest increase in Pellino1 transcripts after stimulation with IL-1β or LPS after 3 hours.

As seen with primary human neutrophils, preliminary qPCR and RT-PCR data from PBECs showed a modest increase in Pellino1 transcripts in response to IL-1β or TNFα stimulation for 1 or 3 hours. Stimulation of PBECs from a different donor with the TLR3 agonist poly(I:C) also showed an increase in Pellino1 after 3 hours of stimulation, however it is important to note that the constitutive levels of Pellino1 between donors was variable. Interestingly, this upregulation of Pellino1 was not seen after stimulation of PBECs with LPS. Pellino2 was not induced after stimulation of PBECs with IL-1β, LPS or TNFα and Pellino3a and Pellino3b were only expressed in low levels.

In contrast to PBECs, the airway cell-line BEAS-2B cells did not exhibit upregulation of Pellino1 in response to the proinflammatory agonists IL-1β, poly(I:C), TNFα or RV1B infection but show a slight downregulation of Pellino1. As seen in PBECs, Pellino2 was not induced after stimulation of PBECs with IL-1β, LPS or TNFα and Pellino3a was only expressed in low levels. Preliminary RT-PCR data suggests that Pellino3b is reduced in response to pro-inflammatory mediators, which supports its suggested role as a negative regulator (Xiao, Qian et al. 2008).

4 Chapter 4 – Results. Creating Pellino1 and MyD88 stable knockdown cells

4.2 Aims

The aim of this chapter was to design a lentivirus that was capable of infecting both dividing and non-dividing cells with a short-hairpin RNA (shRNA) that targets Pellino1 or MyD88 (a functional control) mRNA for degradation through the RNAi pathway. At the commencement of this thesis, available literature investigating the function of Pellino1 suggested that it was important in IL-1 signalling (Jiang, Johnson et al. 2003) and thus we wanted to compare stable Pellino1 knockdown cells with that of a MyD88 knockdown control to compare the phenotype of Pellino1 knockdown cells to that of a known key regulator of the TLR/IL-1R signalling pathway.

The creation of stable knockdown Pellino1 and MyD88 cell-lines was achieved using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System (Invitrogen). Cells that had integrated the lentiviral plasmid into their chromosomes were selected and these cells were single-cell cloned to ensure homogeneous stable production of the shRNA, which leads to target gene knockdown.

4.3 Generating stable Pellino1 and MyD88 knockdown cell-lines

Single-stranded oligos that target Pellino1 or MyD88 were designed using BLOCK-iT™ RNAi Designer (Invitrogen), which designed oligos that were 19-29 nucleotides in length; specific to the target genes; avoid runs of 4 or more of the same nucleotide; and had a low G/C content (below 50%). The sets of primers designed for Pellino1 and MyD88 can be found in **Table 2.6**.

The single-stranded oligos were annealed together and the resulting double-stranded oligo (dsOligo) was then cloned into pENTR/H1/TO. Plasmids that had incorporated the correct insert were tested for knockdown capability in HeLa cells by transiently transfecting the plasmid as outlined in **2.9**. Plasmid 5 for Pellino1 and Plasmid 1 for MyD88 exhibited the best knockdown capability and were chosen to be cloned into the destination vector pLenti4-BLOCKiT™-DEST (**Figure 4.1**). The pLenti4/BLOCK-iT™ expression construct was then transformed into One Shot® Stbl3™ competent *E. coli* as this strain is particularly well-suited for use in cloning unstable DNA such as lentiviral DNA containing direct repeats.

Lentiviral stocks were produced in HEK 293FT cells and were concentrated using the MiniMate™ tangential flow system. Concentrated virus was titred using crystal violet solution and resultant virus was used to transiently transduce HeLa and BEAS-2B cells to test the knockdown capability of each lentivirus.

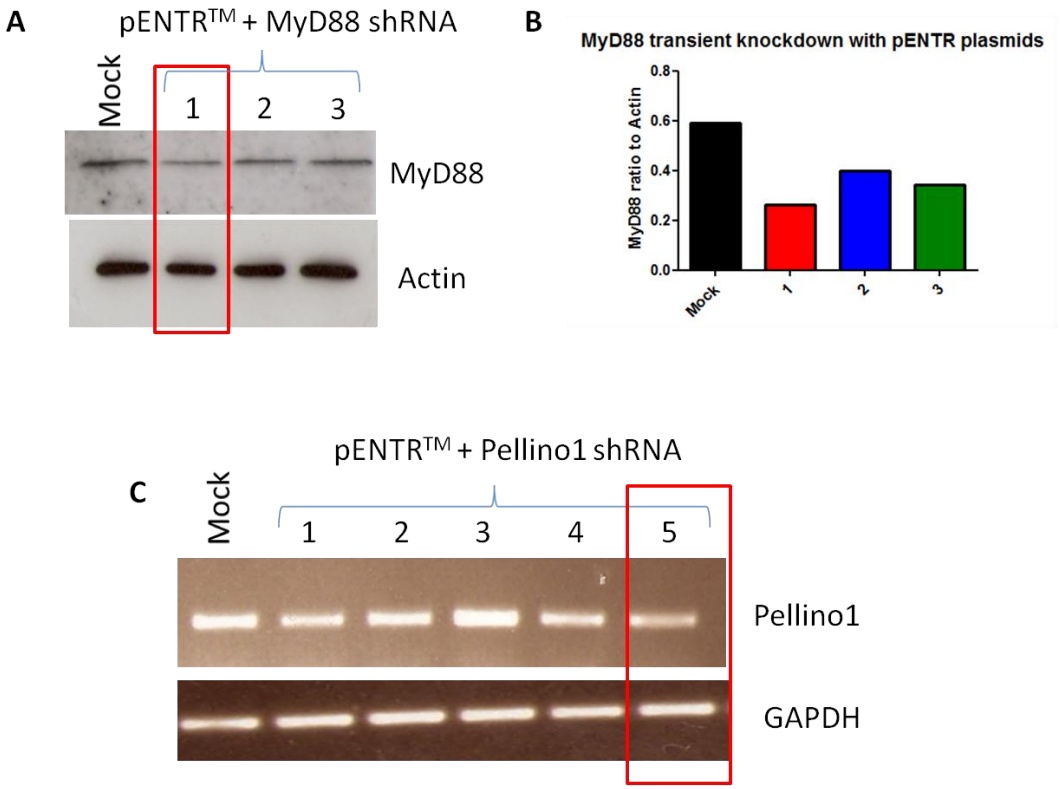


Figure 4.1: Transient knockdown of Pellino1 and MyD88 with pENTR™ plasmids carrying shRNA

Antibiotic resistant colonies that had incorporated the pENTR™ plasmid into their chromosomes with the correct dsOligo insertion were screened for knockdown capability by transiently transfecting HeLa cells. Plasmid 1 for MyD88 showed the best protein knockdown (immunoblot **A** and densitometry **B**) and plasmid 5 for Pellino1 showed the best mRNA knockdown (**C**).

4.4 Transient transduction of Pellino1 and MyD88 shRNA using lentiviral vectors in HeLa and BEAS-2B cells

To determine whether lentiviral particles carrying the shRNA of interest were capable of transducing cells and leading to gene silencing, a transient knockdown experiment using varying multiplicity of infection (M.O.I) was performed. The human cell-line HeLa was used as a control cell-line as these cells are readily transducible and the lung airway epithelial cell-line BEAS-2B was used to investigate the role of Pellino1 within the airway. HeLa and BEAS-2B cells were transduced with lentivirus carrying shRNA either targeting Pellino1 or MyD88 at an M.O.I of 1 or 5.

RT-PCR was used to visualise Pellino1 and MyD88 gene knockdown and it was found that each lentivirus was capable of knocking down their specific target at the mRNA level using a M.O.I of 5 (**Figure 4.2**).

4.5 Single cell cloning and analysis of knockdown

After initial transient transduction experiments were carried out to ensure the lentiviral particles were capable of knocking down the target gene, these particles were then used to generate stable gene knockdown cell-lines by transducing HeLa and BEAS-2B cells with either the lentivirus carrying Pellino1- or MyD88-targetting shRNA at an M.O.I of 5.

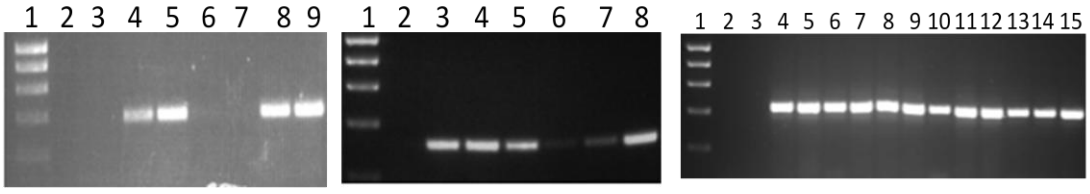
Out of 16 clones of BEAS-2B that had been transduced with Pellino1 shRNA, clones 2, 3, 4, 5, 10, 12, and 15 showed possible Pellino1 mRNA knockdown (**Figure 4.3a+b**) and clone 8 was discarded as it failed to grow. Out of 16 clones of BEAS-2B that had been transduced with MyD88 shRNA, clones 1, 7, 8, 10, 12 and 16 showed possible MyD88 mRNA knockdown (**Figure 4.3c+d**). HeLa clones 8, 9 and 12 that had been transduced with Pellino1 shRNA showed possible gene knockdown however a high proportion of these cells failed to grow and clones 1, 3, 7, 10, 11, 13, and 14 were discarded (**Figure 4.4a+b)**. HeLa clones 3, 6, 7 and 12 that had been transduced with MyD88 shRNA showed possible knockdown at the mRNA level (**Figure 4.4c+d**) and clones 1 and 10 failed to grow.

4.6 Verification of mRNA knockdown using qPCR

Up to 3 different clones from each stable knockdown line that exhibited possible target gene silencing when expression levels were measured using RT-PCR, were taken forward for verification using quantitative PCR (qPCR). BEAS-2B clones 2, 12 and 15 were chosen from the Pellino1 stable knockdown lines and clones 8 and 10 from the MyD88 stable

knockdown lines. HeLa clone 12 was chosen from the Pellino1 stable knockdown lines, however the HeLa MyD88 stable knockdown lines were not analysed further in this project.

Pellino1 mRNA levels were reduced in Clones 2 and 12 BEAS-2B stable Pellino1 knockdown lines, however Clone 15 did not exhibit Pellino1 knockdown (**Figure 4.5a**). Both Clones 8 and 10 for the BEAS-2B MyD88 stable knockdown lines showed target gene silencing at the mRNA level (**Figure 4.5b**). Pellino1 mRNA levels were also reduced in Clone 12 of HeLa stable Pellino1 knockdown (**Figure 4.5c**).



Gel A - **Pellino1**
1.Markers
2.–ve RT no template control
3.–ve PCR no template control
4.Utx HeLa
5.P1 MOI 1 HeLa
6.P1 MOI 5 HeLa
7.P1 MOI 5 BEAS 2B
8.P1 MOI 1 BEAS 2B
9.Utx BEAS 2B

Gel B Lanes - **MyD88**
1.Markers
2.no template control
3.Utx HeLa
4.MyD88 MOI 1 HeLa
5.MyD88 MOI 5 HeLa
6.MyD88 MOI 5 BEAS 2B
7.MyD88 MOI 1 BEAS 2B
8.Utx BEAS 2B

Gel C Lanes - **GAPDH**
1.Markers
2.–ve RT
3.–ve PCR
4.Utx HeLa P1
5.P1 MOI 1 HeLa
6.P1 MOI 5 HeLa
7.Utx HeLa MyD88
8.MyD88 MOI 1 HeLa
9.MyD88 MOI 5 HeLa
10.Utx BEAS 2B P1
11.P1 MOI 1 BEAS 2B
12.Peli 1 MOI 5 BEAS 2B
13.Utx BEAS 2B MyD88
14.MyD88 MOI 1 BEAS 2B
15.MyD88 MOI 5 BEAS 2B

Figure 4.2: Transient knockdown of Pellino 1 and MyD88 in HeLa and BEAS-2B cells

HeLa and BEAS-2B cells were transduced with lentivirus carrying shRNA designed to target Pellino1 (P1) or MyD88 mRNA, with a multiplicity of infection (MOI) of 1 or 5. The shRNA leads to degradation of the specific mRNA through the RNAi pathway, thus knocking down the gene. Messenger RNA was collected using tri-reagent 48 hours after transduction and Pellino1/MyD88 specific PCR primers used to assess knockdown by RT-PCR. **A** Pellino1 lentivirus at a MOI 5 was sufficient to knockdown the specific gene in both HeLa and BEAS-2B cells. **B** MyD88 lentivirus at a MOI 5 was sufficient to knockdown the specific gene in both HeLa and BEAS-2B cells. **C** GAPDH loading control of Pellino1 and MyD88 knockdowns in HeLa and BEAS-2B cells.

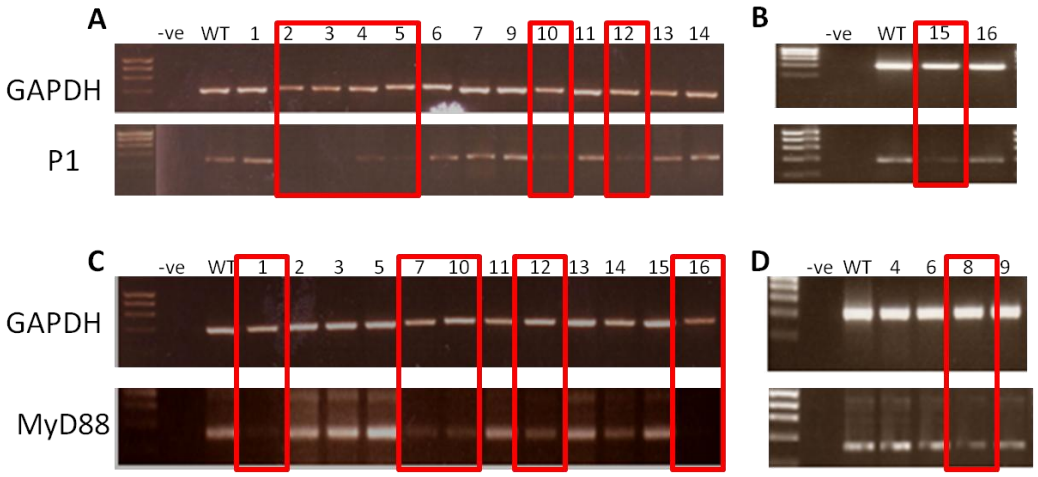


Figure 4.3: Stable transduction of Pellino1 and MyD88 shRNA in BEAS-2B cells

BEAS-2B cells were transduced with Pellino1 (P1) or MyD88 shRNA using a lentiviral vector at a MOI of 5. Cells that had incorporated the pLenti4 expression construct into their chromosome were selected for using Zeocin™. Antibiotic resistant clones were single cell cloned to gain homogeneous populations and up to 16 clones were picked from each transduction to analyse for gene knockdown. Gels show BEAS-2B cells transduced with Pellino1 shRNA (**A+B**) and MyD88 shRNA (**C+D**). Numbers at the top of the gel correspond to clone number and red boxes highlight possible gene knockdown.

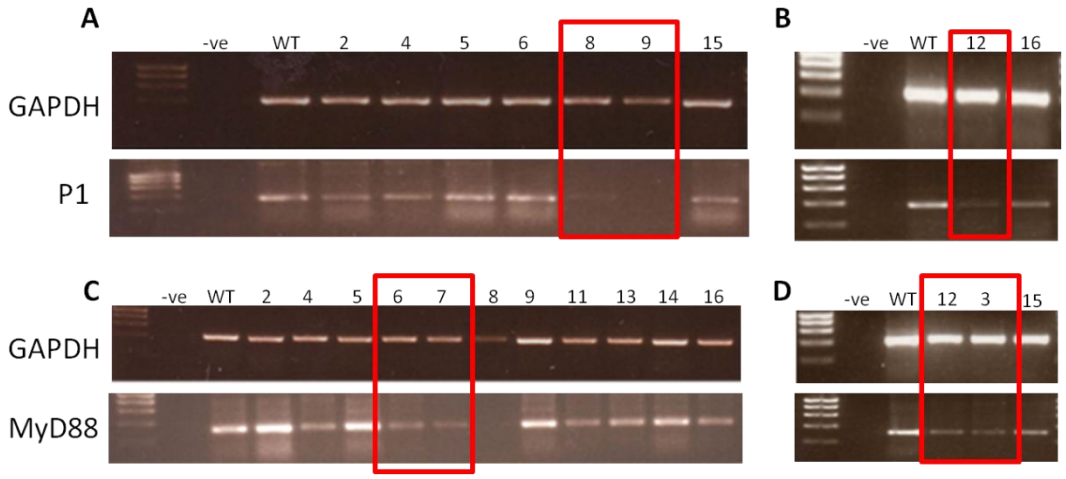


Figure 4.4: Stable transduction of Pellino1 and MyD88 shRNA in HeLa cells

HeLa cells were transduced with Pellino1 (P1) or MyD88 shRNA using a lentiviral vector at a MOI of 5. Cells that had incorporated the pLenti4 plasmid in to their chromosome were selected for using zeocin™. Antibiotic resistant clones were single cell cloned to gain homogeneous populations and up to 16 clones were picked from each transfection to analyse for gene knockdown. Gels show HeLa cells transfected with Pellino1 shRNA (**A+B**) and MyD88 shRNA (**C+D**). Numbers at the top of the gel correspond to clone number and red boxes highlight possible gene knockdown.

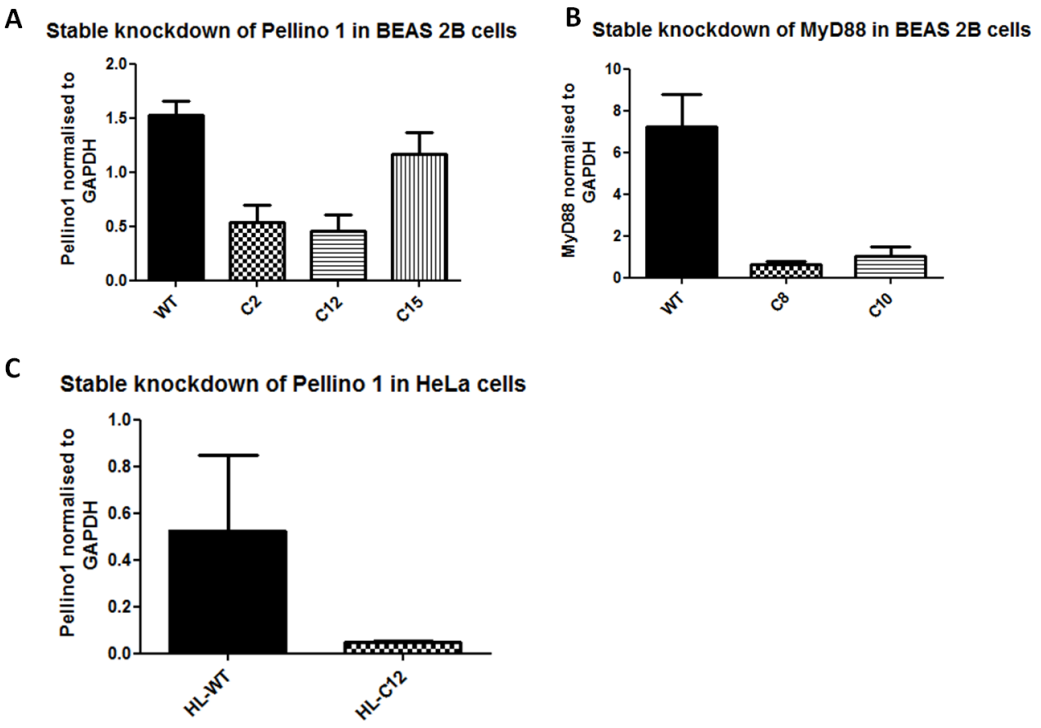


Figure 4.5: Pellino1 and MyD88 stable knockdown in HeLa and BEAS-2B cells

Pellino1 mRNA relative expression levels were measured using qPCR in BEAS-2B clones 2 (C2), clone 12 (C12) and clone 15 (C15) stable Pellino1 knockdown cell lines compared to that of wild-type BEAS-2B (WT) (**A**). MyD88 mRNA relative expression levels were measured using qPCR in BEAS-2B clones 8 (C8) and clone 10 (C10) stable MyD88 knockdown cell-lines compared to that of wild-type BEAS-2B (WT) (**B**). Pellino1 mRNA relative expression levels were measured using qPCR in HeLa clone 12 (HL-C12), clone 12 stable Pellino1 knockdown cell-line compared to that of wild-type HeLa cells (HL-WT) (**C**). Data are expressed as mean±SEM from 3 independent experiments.

4.7 Functional analysis of BEAS-2B MyD88 stable knockdown clone 8

MyD88 is a key mediator of the TLR/IL-1R signalling pathway and has been used as both a proof-of-principle for the generation of knockdown BEAS-2B cell-lines using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System and also as a functional control for Pellino1 knockdown cells.

The effect of MyD88 knockdown on induced cytokine release in BEAS-2B cells was assessed using the stable MyD88 knockdown clone 8 (B2B MyD88). B2B MyD88 clone and wild-type BEAS-2B cells were stimulated with a dose response of IL-1β, poly(I:C) and TNFα for 24 hours and IL-8 generation in cell-free supernatants was measured using ELISA. Responses to IL-1β and TNFα were measured as a positive and negative control, respectively, as MyD88 has previously been shown to be key mediator in IL-1R signalling but is thought to be independent of TNFR1 signalling. The involvement of MyD88-dependent signalling in response to viral stimuli in human airway cells was largely uncharacterised at the commencement of this thesis and was a keen interest within our group. We used the poly(I:C) to investigate the role of MyD88 in TLR3 signalling.

As expected, B2B MyD88 showed significantly reduced IL-8 generation in response to IL-1β (**Figure 4.6a**) and response to TNFα was unaffected in B2B MyD88 cells (**Figure 4.6c**). IL-8 generation was also unaffected in response to poly(I:C) (**Figure 4.6b**). RNA was also extracted from these cells to measure MyD88 mRNA expression levels by qPCR in the B2B MyD88 clone compared to that of wild-type BEAS-2B cells; MyD88 remained knocked down in the B2B MyD88 clone throughout the functional experiments (**Figure 4.6d**).

Due to time constraints and the continued interest in Pellino1, further characterisation of the MyD88 knockdown BEAS-2B cell-line was carried out by a colleague in our group. It was found that infection of MyD88 knockdown BEAS-2B cells with the human viral pathogen RV1B led to a significant reduction in IL-8 generation. This finding may be due to decreased responses to autocrine IL-1 generation in MyD88 knockdown cells, since pre-incubation of BEAS-2B cells with IL-1ra also led to the significant reduction of IL-8 production. It is also interesting to note that MyD88 also played a key role in antiviral immunity in response to rhinoviral infection, as MyD88 knockdown led to a significant increase in RV1B replication (Stokes, Ismail et al. 2011).

These data suggest that knockdown of key regulators of the TLR/IL-1R signalling pathway can provide insights into the signalling pathways that control inflammation and generation of stable knockdown cell-lines is an effective way to investigate these pathways. The successful generation of the MyD88 knockdown BEAS-2B cell-line led us to believe that the BLOCK-iT™ Inducible H1 Lentiviral RNAi System was a reliable and effective method for the generation of Pellino1 knockdown cell-lines.

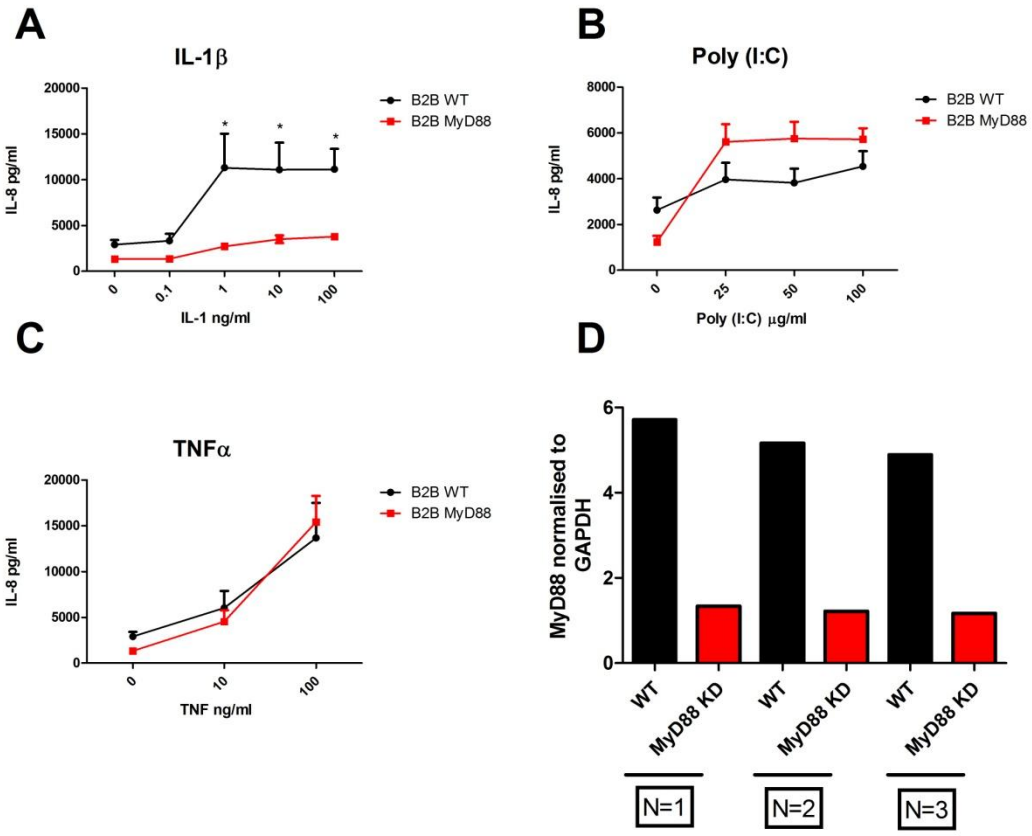


Figure 4.6: IL-8 generation in response to IL-1β, poly(I:C) and TNFα stimulation in MyD88 stable knockdown BEAS-2B Clone 8

BEAS-2B wild-type (B2B WT) and MyD88 stable knockdown clone 8 (B2B MyD88) cells were treated for 24 hours with a concentration response of IL-1β (**A**), TNFα (**B**), poly(I:C) (**C**) or media (**A-C**). Supernatants were collected and analysed for IL-8 generation by ELISA. Data are expressed as mean±SEM from 6 independent experiments from 3 different passages. MyD88 knockdown at the mRNA level for each passage are expressed as MyD88 levels that have been normalised to control GAPDH levels (**D**). Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test, comparing B2B WT vs. B2B MyD88 (*$p<0.05$).

4.8 Functional analysis of HeLa Pellino1 stable clone 12

The successful creation of a MyD88 knockdown BEAS-2B cell-line suggested that the BLOCK-iT™ Inducible H1 Lentiviral RNAi System was a good method to generate effective stable knockdown cell-lines. Therefore we utilised this method to investigate the role of Pellino1 in human cell-lines. We initially used human HeLa cells as this robust cell-line is readily transducible and functional data could be compared with that of Pellino1 stable knockdown in lung airway epithelial cells.

The effect of Pellino1 knockdown on induced-cytokine release in HeLa cells was assessed using the stable Pellino1 knockdown clone HL-C12. HL-C12 clone and wild-type HeLa cells were stimulated with different doses of specific cytokines for 24 hours and IL-8 generation in cell-free supernatants was measured using ELISA. The function of Pellino1 in specific signalling pathways controlling inflammation was tested by stimulating Pellino1 knockdown cells with IL-1β, poly(I:C) and TNFα. These stimuli were chosen as Pellino1 has previously been shown to be involved in both the IL-1 (Jiang, Johnson et al. 2003) and TLR3 (Chang, Jin et al. 2009) signalling pathways. TNFα was used as a control for non-specific effects of the transduction process, since this cytokine stimulates an unrelated signalling pathway to TIR signalling, which Pellino1 has not previously been implicated in.

HL-C12 showed a modest increase in IL-8 generation in response to IL-1β and poly(I:C) compared to that of wild-type HeLa cells (**Figure 4.7a+b**), however IL-8 generation in response to TNFα was unaffected in HL-C12 cells (**Figure 4.7c**). Data are expressed as amount of IL-8 per 10,000 cells to account for the differences in cell growth rate between HL-C12 clone and wild-type HeLa cells. RNA was also extracted from these cells to measure Pellino1 mRNA expression levels by qPCR in the HL-C12 clone compared to that of wild-type HeLa cells; Pellino1 remained knocked down in the HL-C12 clone throughout the functional experiment (**Figure 4.7d**). These data suggest that the lentivirus carrying the shRNA targeting Pellino1 that was generated using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System was capable of creating stable Pellino1 knockdown cell-lines and thus was used to transduce our cell-type of interest, the lung airway epithelial cell-line, BEAS-2B.

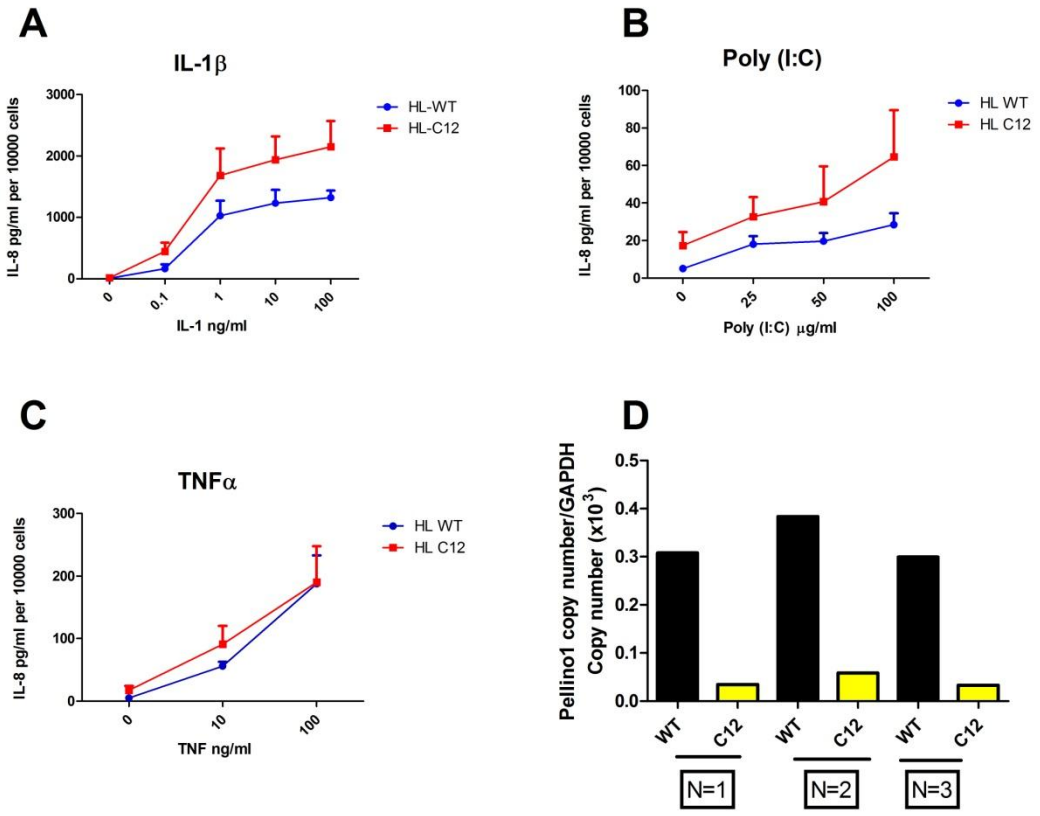


Figure 4.7: IL-8 generation in response to IL-1β, poly(I:C) and TNFα stimulation in Pellino1 stable knockdown HeLa Clone 12

HeLa wild-type (WT) and Pellino1 stable knockdown (HL-C12) cells were treated for 24 hours with a concentration response of IL-1β (**A**), TNFα (**B**), poly(I:C) (**C**) or media (**A-C**). Supernatants were collected and analysed for IL-8 generation by ELISA. Data are expressed as mean±SEM of IL-8 generation per 10000 cells from 6 independent experiments from 3 different passages. Pellino1 knockdown at the mRNA level for each passage are expressed as the ratio of Pellino1 copy number to control GAPDH copy number (**D**). The difference in cytokine generation between HL-C12 and WT was not statistically significant using two-way ANOVA with Bonferroni post-tests.

4.9 Functional analysis of BEAS-2B Pellino1 stable knockdown clones 2 and 12

Successful generation of BEAS-2B MyD88 and HeLa Pellino1 stable knockdown clones suggested that the lentivirus carrying the shRNA targeting Pellino1 was capable of knocking down Pellino1 and that BEAS-2B cells were an efficient cell-type to generate stable cell-lines using this method. Following this, we wanted to create Pellino1 stable knockdown BEAS-2B clones to investigate the importance of Pellino1 in the signalling pathways controlling inflammation in airway epithelial cells.

The effect of Pellino1 knockdown on induced-cytokine release in BEAS-2B cells was assessed using the stable Pellino1 knockdown clones P1-C2 and P1-C12, which were the two clones that exhibited the most efficient knockdown during verification of the clones. Clones P1-C2 and P1-C2 were stimulated alongside the control cells [wild-type BEAS-2B (B2B-WT) and a GFP-expressing stable BEAS-2B cell line that had also been created using a lentiviral vector and had under-gone single cell cloning (B2B-GFP)], with a dose response of IL-1β, poly(I:C) and TNFα for 24 hours. These agonists were chosen for the same reasons as outlined in **section 4.7** and these agonists were used in previous experiments using HeLa Pellino1 stable knockdown cells and therefore functional outputs could be compared between cell-types.

IL-8 generation in cell-free supernatants was measured using ELISA. P1-C2 generated comparable amounts of IL-8 to that of wild-type BEAS-2B in response to IL-1β (**Figure 4.8a**), poly(I:C) (**Figure 4.8b**) and TNFα (**Figure 4.8c**). However, in contrast to this, P1-C12 generated much higher levels of IL-8 in response to IL-1β (**Figure 4.8a**), poly(I:C) (**Figure 4.8b**) and TNFα (**Figure 4.8b**). Interestingly, although the GFP-expressing stable BEAS-2B cell line produced similar amounts of IL-8 in response to poly(I:C) and TNFα as wild-type BEAS-2B, its responses to IL-1β were significantly higher (**Figure 4.8a**). Data are expressed as amount of IL-8 per 10,000 cells to account for the differences in cell growth between P1-C2, P1-C12, B2B-GFP and B2B-WT cells. RNA was also extracted from these cells to measure Pellino1 mRNA expression levels by qPCR in the P1-C2 and P1-C12 clone compared to that of wild-type BEAS-2B cells; mRNA analysis showed that Pellino1 knockdown was modest during these experiments (**Figure 4.8d**).

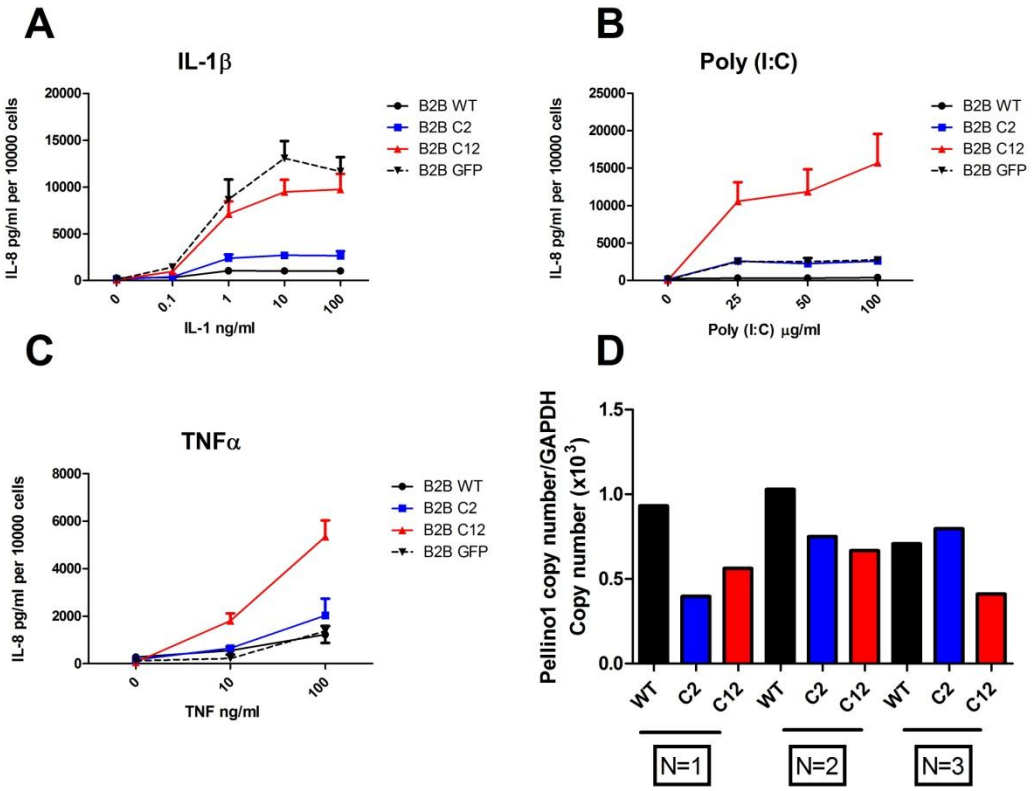


Figure 4.8: IL-8 generation in response to IL-1β, poly(I:C) and TNFα stimulation in Pellino1 stable knockdown BEAS-2B Clone 2 and 12

BEAS-2B wild-type (B2B WT) and Pellino1 stable knockdown clone 2 (B2B-C12) and clone 12 (B2B-C12) cells were treated for 24 hours with a concentration response of IL-1β (**A**), TNFα (**B**), poly(I:C) (**C**) or media (**A-C**). Supernatants were collected and analysed for IL-8 generation by ELISA. Data are expressed as mean±SEM of IL-8 generation per 10000 cells from 6 independent experiments from 3 different passages. Pellino1 knockdown at the mRNA level for each passage are expressed as the ratio of Pellino1 copy number to control GAPDH copy number (**D**).

4.10 Pellino1 knockdown over passage

Pellino1 knockdown in BEAS-2B stable clones 2 and 12 only exhibited modest gene knockdown during functional experimentation despite efficient Pellino1 knockdown being measured during original verification of the clones. This would suggest that BEAS-2B Pellino1 stable knockdown clones 2 and 12 exhibited variable levels of knockdown. To test whether knockdown was dependent on passage number, BEAS-2B clone 2 (B2B-C2), clone 12 (B2B-C12) and HeLa clone 12 (HL-C12) were passaged up to 10 times and Pellino1 mRNA levels were analysed after each passage. Both Pellino1 stable knockdown clone 2 and clone 12 exhibited less Pellino1 mRNA knockdown at the later passage numbers (**Figure 4.9a+b**), whereas stable Pellino1 knockdown in HeLa clone 12 showed consistent Pellino1 mRNA knockdown (**Figure 4.9c**).

B2B-C2 and B2B-C12 not only exhibit inconsistent Pellino1 knockdown but also behave very differently in respect to signalling pathways that control inflammation, despite being clones of the same transduction experiment. In the absence of a clear understanding of the role of Pellino1 in these pathways it is difficult to determine which clone is a true representative of the Pellino1 knockdown phenotype in airway epithelial cells. Considering this, analysis of these clones was not pursued any further and a different system, Sigma MISSION^{TM} system, was used to generate new Pellino1 stable knockdown BEAS-2B clones.

4.11 Pellino1 stable knockdown using the Sigma MISSION™ system

Creating a stable Pellino1 knockdown using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System was hitherto unsuccessful; therefore a different system was then utilised for the creation of Pellino1 knockdown BEAS-2B. The Sigma MISSION^{TM} system provides 4 ready-made lentiviral vectors each containing a different shRNA targeting Pellino1 (**Appendix 1**). Transient transduction of BEAS-2B cells with each lentivirus at an M.O.I of 1 showed that virus 2 showed the most effective Pellino1 knockdown at the mRNA level (**Figure 4.10**) and this virus was used to generate a stable Pellino1 knockdown line in BEAS-2B cells.

Virus 2 was used to generate Pellino1 stable knockdown BEAS-2B cell-lines and ten clones from each M.O.I were analysed for target gene knockdown at the mRNA level. None of the clones exhibited Pellino1 knockdown and therefore could not be analysed further (**Figure 4.11**).

Generation of stable Pellino1 knockdown cell-lines in BEAS-2B cells proved difficult as both the BLOCK-iT™ Inducible H1 Lentiviral RNAi System and the Sigma MISSION™ system were unable to yield clones that exhibited consistent target gene knockdown. This may be due to an essential role of Pellino1 in normal cellular functions and this could be represented by the fact that clones exhibiting antibiotic resistance were also slow growing, perhaps suggesting a role for Pellino1 in cell growth. To overcome this, and other limitations of an appropriate control cell-line, we wanted to generate a Tet repressor expressing cell-line for inducible shRNA gene knockdown in BEAS-2B cells.

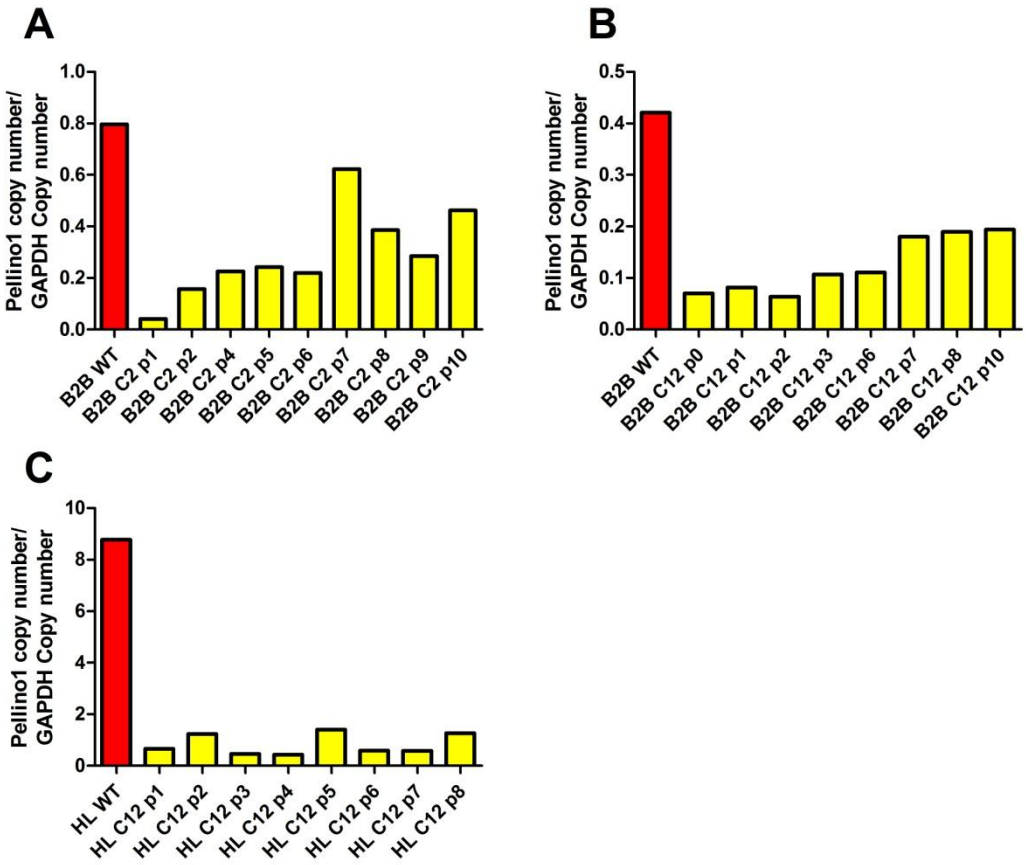


Figure 4.9: Passage dependent Pellino1 knockdown in BEAS-2B clones 2 and 12 and HeLa Clone 12

Pellino1 knockdown at the mRNA level was measured using qPCR from RNA that was extracted from Pellino1 stable knockdown BEAS-2B clone 2 (B2B-C2) (**A**), clone 12 (B2B-C12) (**B**) and HeLa clone 12 (HL-C12) (**C**) after each passage up to passage 8-10. Data are expressed as a ratio of Pellino1 copy number to control GAPDH copy number.

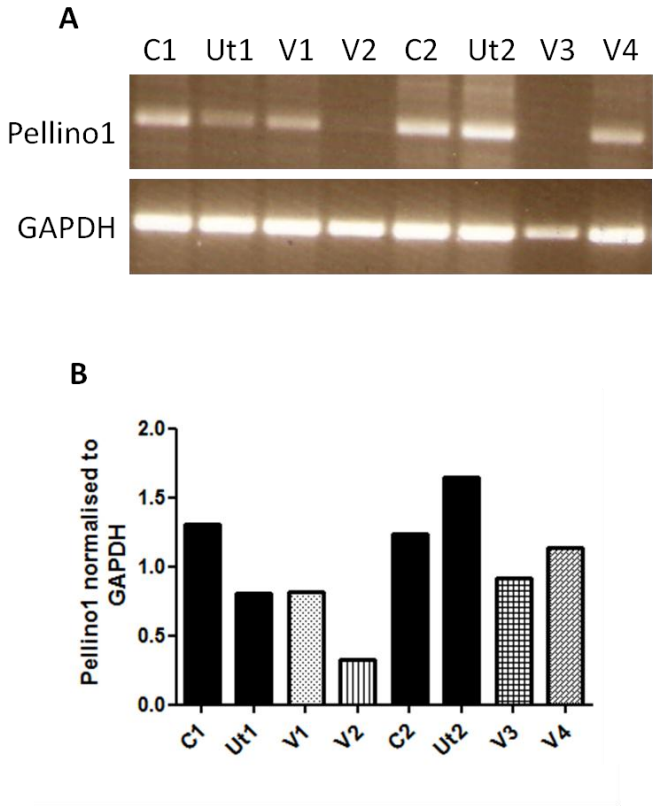


Figure 4.10: Transient Pellino1 mRNA knockdown using Sigma MISSION™ lentiviral particles in BEAS-2B cells

RNA was extracted from BEAS-2B cells that were either control (C1, C2) untransduced (Ut1, Ut2), or transduced with a Sigma MISSION™ lentiviral particle carrying a Pellino1-targeting shRNA (V1, V2, V3, V4). Pellino1 knockdown at the mRNA level was measured using RT-PCR (**A**) and qPCR (**B**) and viral particle 2 (V2) exhibited the best degree of Pellino1 knockdown.

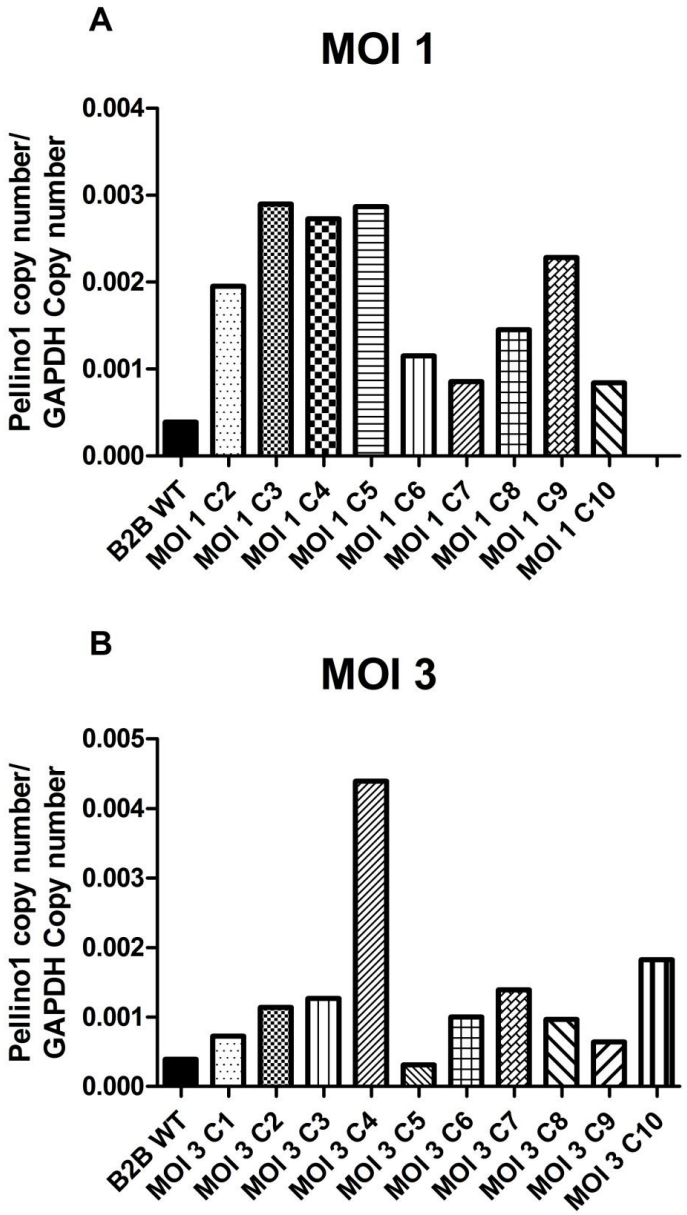


Figure 4.11: Stable Pellino1 knockdown using the Sigma MISSION™ system in BEAS-2B cells

BEAS-2B cells were transduced using Virus 2 of the Sigma MISSION™ system at an M.O.I of 1 and 3 and cells that had stably incorporated the shRNA-carrying construct into the chromosome were selected for using the puromycin selection marker. Ten clones from each M.O.I were analysed for Pellino1 mRNA knockdown using qPCR. Clones (C1 – C10) that had been transduced at an M.O.I of 1 (**A**) or 3 (**B**) did not show Pellino1 knockdown compared to wild-type BEAS-2B cells (B2B WT).

4.12 Tet repressor line

As previous stable Pellino1 knockdown in BEAS-2B cells were unsuccessful using both the BLOCK-iT™ Inducible H1 Lentiviral RNAi System and the Sigma MISSION™ system, a Tet Repressor BEAS-2B cell line was to be generated to allow for inducible shRNA production leading to Pellino1 knockdown. This was another feature of the BLOCK-iT™ Inducible H1 RNAi system and would provide a better control cell-line than the previously described wild-type or GFP overexpressing line. Since the Tet Repressor line will have undergone the transduction process, unlike wild-type controls, they would be a homogeneous population of cells, which would control for differences in cytokine responses between clones. The Tet repressor line is regulated by tetracycline, so that only when tetracycline is present can the shRNA be transcribed leading to gene knockdown.

4.12.1 Creating a Tet repressor expressing host cell line

A pLenti6/TR plasmid (plasmid map and features can be found in **Appendix 1**) that expresses the Tet repressor protein was packaged into a lentivirus as outlined in **sections 2.14.4 – 2.14.8.** The resulting lentivirus was used to transiently transduce BEAS-2B cells with an M.O.I of 10 to ensure that the lentivirus was capable of transducing BEAS-2B and producing the Tet repressor protein. As good Tet repressor mRNA expression was detected in cells that had been transduced with an M.O.I of 10 (**Figure 4.12**), this M.O.I was used to create a BEAS-2B cell line that stably produce the Tet repressor protein. After transduction with the lentivirus carrying the pLenti6 plasmid, BEAS-2B cells that had incorporated the construct into the chromosome were selected for using blasticidin, however despite successful transient transduction of the pLenti6/TR plasmid, no cells exhibited antibiotic resistance.

4.12.2 Troubleshooting Tet repressor transduction

Transduction with the lentivirus carrying the pLenti6 construct that encodes the Tet repressor protein failed to produce any antibiotic resistant clones, despite effective transient transduction of BEAS-2B cells (**Figure 4.12**). Initially, a blasticidin kill curve experiment was repeated to verify the appropriate amount of antibiotic required to select for transduced BEAS-2B cells. Five μg/ml blasticidin, which had been used in previous experiments, was confirmed as the appropriate concentration. To investigate whether the resistance gene had been lost during lentiviral production, RT-PCR primers specific to the blasticidin resistance gene were used to test whether upon transduction with the lentivirus carrying pLenti6, the blasticidin resistance maker is present in the transduced BEAS-2B

cells. It was found that blasticidin resistance gene (*bsd* from *Aspergillus terreus*) was present in BEAS-2B cells that had been transduced with pLenti6 and not in untransduced BEAS-2Bs (**Figure 4.13**).

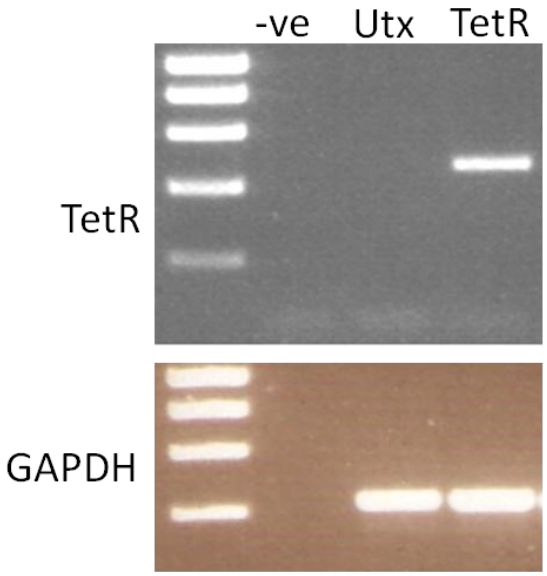


Figure 4.12: Transient transfection of Tet repressor using lentiviral vector in BEAS-2B cells

BEAS-2B cells were transduced with lentivirus carrying pLenti6, a plasmid encoding the Tet repressor protein, at a MOI of 10. Forty eight hours after transduction, RNA was extracted and expression of the Tet repressor was measured by RT-PCR.

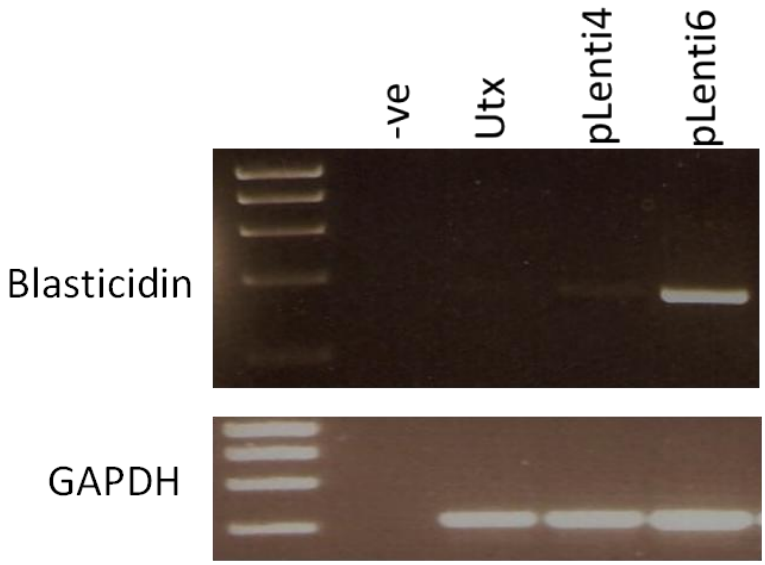


Figure 4.13: Testing the presence of the blasticidin resistance gene in transduced BEAS-2B cells

RNA was extracted from BEAS-2B cells that were either untransduced (Utx), or transduced with pLenti4-expression construct (pLenti4) or pLenti6/TR (pLenti6) construct. RT-PCR was used to detect mRNA levels of the blasticidin resistance gene (*bsd* from *Aspergillus terreus*) and the control gene GAPDH. As expected, the blasticidin resistance gene mRNA expression could not be detected in the untransduced or pLenti4 transduced BEAS-2B cells, but was present in the pLenti6/TR transduced cells.

4.13 Summary

The aim of this chapter was to generate Pellino1 and MyD88 stable knockdown cell-lines in both HeLa and BEAS-2B cells using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System. A lentiviral vector carrying shRNA specific to either Pellino1 or MyD88 were manufactured and used to transduce HeLa and BEAS-2B cells and untransduced cells were selected out using the antibiotic resistance marker. After which, these cells were single-cell cloned to produce cell-lines that exhibited a consistent level of target gene knockdown.

Transduction of the Pellino1 or MyD88 shRNA using the specific lentiviral vector generated transient target gene knockdown in both HeLa and BEAS-2B cells and several clones from each cell-line exhibited target gene knockdown (measured by RT-PCR). Gene knockdown was verified using qPCR in BEAS-2B clones 2, 12 and 15 for Pellino1 knockdown and clones 8 and 10 for MyD88 knockdown and HeLa Pellino1 knockdown clone 12. Pellino1 stable knockdown BEAS-2B clones 2 (B2B–C2) and 12 (B2B–C12), MyD88 stable knockdown BEAS-2B clone 8 (B2B–C8) and HeLa Pellino1 stable knockdown clone 12 (HL–C12) exhibited the highest level of target gene knockdown and were used for functional experiments.

The BEAS-2B MyD88 cell-line was created as both a proof-of-principle of stable knockdown generation and a functional control for Pellino1 knockdown in lung airway cells. As expected, this clone exhibits significantly reduced IL-8 generation in response to IL-1β and preserved IL-8 in response to TNFα. Our group is interested in how the TLR/IL-1R signalling pathway contributes to inflammatory signalling associated with viral infection and despite IL-8 production being unchanged in the MyD88 knockdown clone in response to poly(I:C), further work carried out in our lab on this clone has shown that MyD88 plays a key role in antiviral responses to rhinovirus infection (Stokes, Ismail et al. 2011).

Stable Pellino1 knockdown clones were initially generated using the human HeLa cells as this cell-line is easily susceptible to genetic manipulation. Stable Pellino1 knockdown in HeLa cells led to slight increase in IL-8 generation in response to IL-1β and poly(I:C) stimulation, which was also seen in stable Pellino1 knockdown in lung airway cell-line BEAS-2Bs. However, in contrast to HeLa Pellino1 stable knockdown (HL-C12), Pellino1 knockdown in both BEAS-2B clones 2 (B2B–C2) and 12 (B2B–C12) exhibited inconsistent and variable knockdown and B2B–C12 produced much higher levels of IL-8 in response to all proinflammatory agonists tested (IL-1β, poly(I:C) and TNFα) compared to that of B2B–C2 and a GFP-expressing control.

As stable Pellino1 knockdown clones in BEAS-2B created using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System exhibited inconsistent levels of knockdown, a different system, the Sigma MISSION^{TM} system, which provides 4 lentiviruses each carrying a different shRNA targeting Pellino1 was used to generate stable Pellino1 knockdowns in BEAS-2B cells. Initial transient transduction experiments showed that virus 2 led to the highest level of Pellino1 knockdown and this virus was used to create stable knockdown BEAS-2B cell-lines. Despite successful transient Pellino1 knockdown, no clones out of 19 tested exhibited significant Pellino1 knockdown and therefore could not be analysed further.

One of the main limitations associated with using stable knockdown cell-lines for functional experiments is the lack of a suitable control cell-line and this could be overcome by creating an inducible knockdown cell-line by utilising tetracycline-induced shRNA production using a lentivirus carrying pLenti6 which encodes the Tet repressor protein. Cells transduced with pLenti6 did not exhibit any blasticidin resistance despite successful transient transduction of both the Tet repressor and blasticidin resistance sequences.

5 Chapter 5 – Results. Transient knockdown of Pellino1 in airway cells: studying the role of Pellino1 in inflammatory signalling

5.2 Effect of transient knockdown of Pellino1 on cytokine release in response to TLR/IL-1R agonists in HeLa

Attempts to clone a stable Pellino1 knockdown BEAS-2B cell-line were unsuccessful however transient knockdown experiments exhibited a good degree of target gene knockdown at the mRNA level. Short interfering RNA (siRNA) can be introduced into cells by a lipid-based transfection reagent, which leads to gene silencing via the RNAi pathway (see **Section 2.10**). This method was utilised to transiently knockdown Pellino1 in HeLa cells, which led to the significant reduction of Pellino1 mRNA transcripts compared to control and non-targeting (scrambled) siRNA treatments as measured by qPCR (**Figure 5.1**). Pellino1 knockdown and scrambled transfected cells were stimulated with the IL-1R and TLR agonists IL-1β and poly(I:C) and the non-TIR agonist TNFα. Generation of the NF-κB-stimulated gene, IL-8, and the Interferon-stimulated gene, RANTES was measured using ELISA. We measured these cytokines as endpoints for NF-κB and IRF signalling in response to viral stimuli and to investigate the possibility of Pellino1 to selectively regulate the NF-κB signalling arm of these pathways as a process to allow the down-regulation of neutrophilic inflammation whilst retaining antiviral immunity.

We initially knocked down Pellino1 in the human HeLa cells as this cell-line are easily transfectable and could be used as a comparison for Pellino1 knockdown in lung airway cells. Transient Pellino1 knockdown in HeLa cells led to reduced IL-8 generation in response to the TLR3 agonist and viral mimic poly(I:C) (**Figure 5.2b**). IL-8 generation in response to both IL-1β and TNFα was preserved in Pellino1 knockdown HeLa cells (**Figure 5.2a+c**) and the interferon-stimulated gene (ISG) RANTES was reduced only after stimulation with the highest dose of poly(I:C) (100 μg/ml) (**Figure 5.3**).

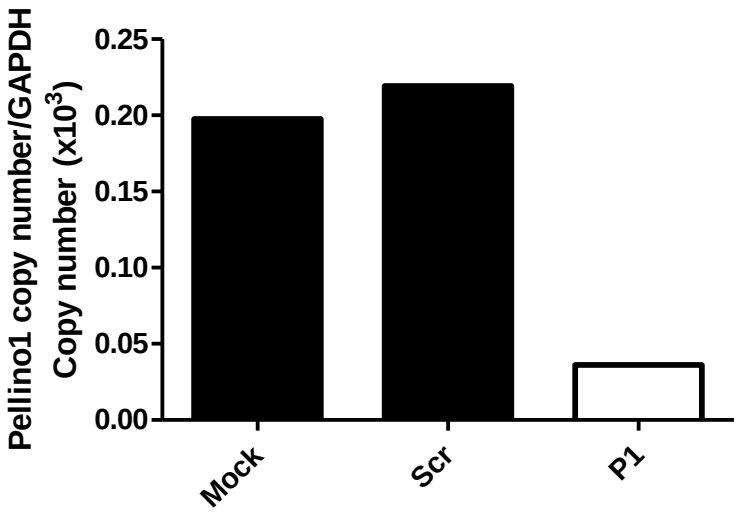


Figure 5.1: Transient Pellino1 knockdown at the mRNA level in HeLa cells

HeLa cells were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA. Total RNA was extracted from these cells 48 hours post-transfection and converted into cDNA. Quantitative RT-PCR (qPCR) was used to compare Pellino1 mRNA expression levels in mock and scrambled siRNA (Scr) transfected to that of Pellino1 knockdown HeLa cells. Pellino1 copy number was normalised to GAPDH copy number as a loading control and data are expressed as the mean from 2 independent experiments.

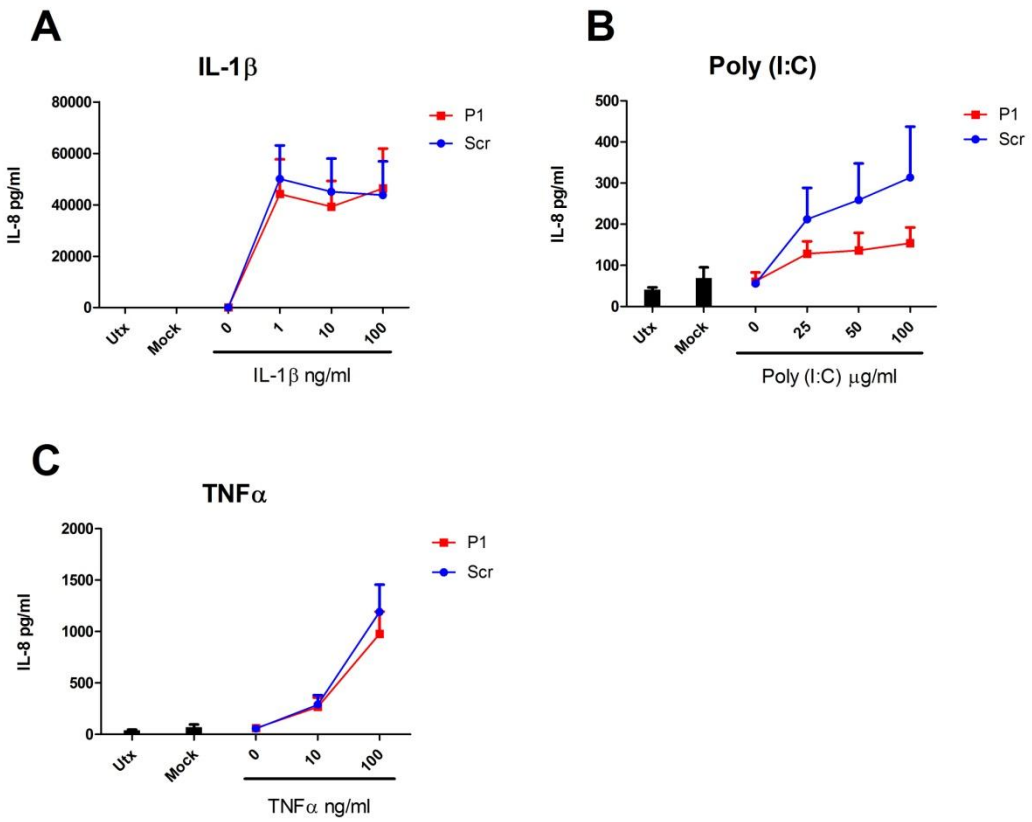


Figure 5.2: IL-8 generation in Pellino1 knockdown HeLa cells in response to IL-1β, poly(I:C) and TNFα stimulation

HeLa cells were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of IL-1β (**A**), poly(I:C) (**B**), TNFα (**C**) or media (**A-C**). Untransfected (Utx) and mock transfected HeLa cells were treated with media only. Supernatants were collected and analysed for IL-8 generation by ELISA. Data are expressed as the mean from 2 experiments.

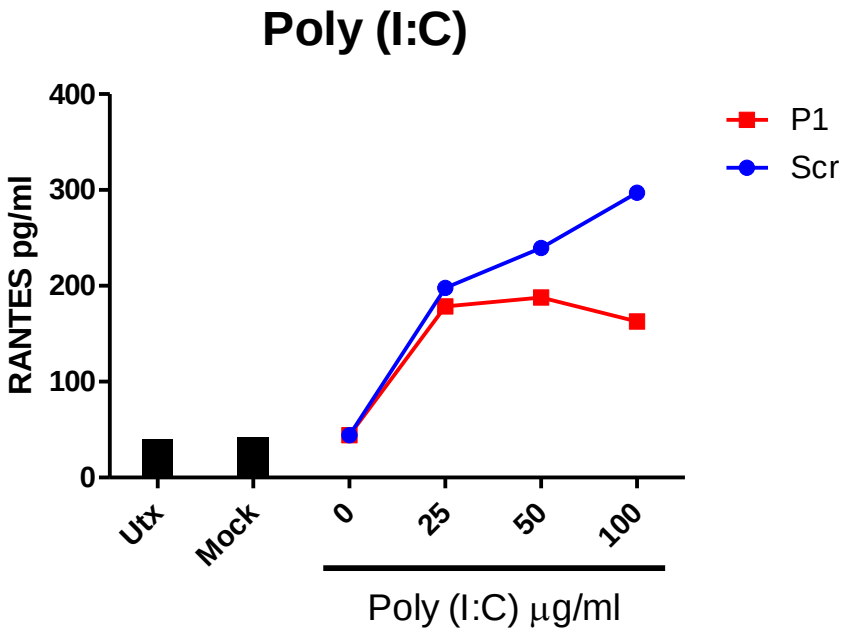


Figure 5.3: RANTES generation in Pellino1 knockdown HeLa cells in response to Poly(I:C) stimulation

HeLa cells were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of poly(I:C). Untransfected (Utx) and mock transfected HeLa cells were treated with media only. Supernatants were collected and analysed for RANTES generation by ELISA. Data are expressed as the mean from 2 independent experiments.

5.3 Effect of transient knockdown of Pellino1 on cytokine release in response to TLR/IL-1R agonists in BEAS-2B

HeLa are an unrepresentative cell-line and we wanted to investigate the functional importance of Pellino1 in lung airway epithelium and so Pellino1 was transiently knocked down in the immortalised lung airway epithelial cell-line BEAS-2B.

Transfection of Pellino1 targeted siRNA led to the significant reduction in Pellino1 transcripts compared to control and scrambled siRNA transfected BEAS-2B, which were measured using qPCR (**Figure 5.4a**). To ensure that knockdown is not overcome in response to pro-inflammatory agonists due to the potential role of Pellino1 in the TLR/IL-1R signalling pathway, the effect of IL-1β, poly(I:C) and TNFα stimulation on Pellino1 mRNA levels in Pellino1 knockdown cells was measured by qPCR. It was found that knockdown was not affected by stimulation with TLR/IL-1R/TNFR agonists (**Figure 5.4b**).

Pellino1 knockdown and scrambled transfected cells were stimulated with IL-1β, poly(I:C) or TNFα. IL-1β was used as Pellino1 has previously been shown to be involved in both the IL-1 (Jiang, Johnson et al. 2003) signalling pathway and poly(I:C) was used as more recent data suggests a role for Pellino1 in TLR3 signalling (Chang, Jin et al. 2009). The latter study was supported by transient knockdown of Pellino1 in HeLa cells that shows a reduction in IL-8 in response to poly(I:C) and therefore we wanted to explore this role in TLR3 signalling in airway epithelial cells. TNFα was used as a control for non-specific effects of the transfection process, since this cytokine stimulates an unrelated signalling pathway to TIR signalling, which Pellino1 has not previously been implicated in.

IL-8 and RANTES generation was measured using ELISA. Transient Pellino1 knockdown in BEAS-2B cells led to significant reduction in IL-8 generation in response to IL-1β and poly(I:C) (**Figure 5.5a+b**). IL-8 generation in response to TNFα was preserved in Pellino1 knockdown BEAS-2B cells (**Figure 5.5c**) and the ISG RANTES was unaffected by Pellino1 knockdown for any agonist tested (**Figure 5.6**).

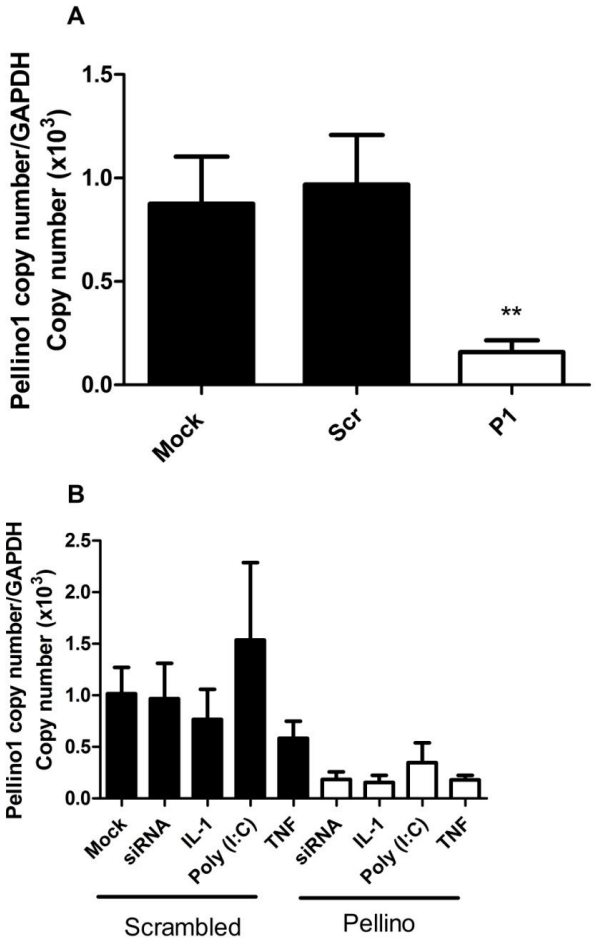


Figure 5.4: Transient Pellino1 knockdown at the mRNA level in BEAS-2B

BEAS-2B cells were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA. Total RNA was extracted from these cells 48 hours post-transfection and converted into cDNA. Quantitative RT-PCR was used to compare Pellino1 mRNA expression levels in mock and scrambled siRNA (Scr) transfected to that of Pellino1 knockdown BEAS-2Bs. (**A**). Pellino1 mRNA expression levels in knockdown BEAS-2B cells stimulated with 100 ng/ml IL-1β, 100 µg/ml Poly(I:C) or 100 ng/ml TNFα were compared to that of mock transfected and scrambled siRNA transfected stimulated with the same agonists using qPCR (**B**). Pellino1 copy number was normalised to GAPDH copy number as a loading control and data are expressed as mean±SEM from 4 (**A**) or 3 (**B**) independent experiments, respectively. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 (**p<0.01).

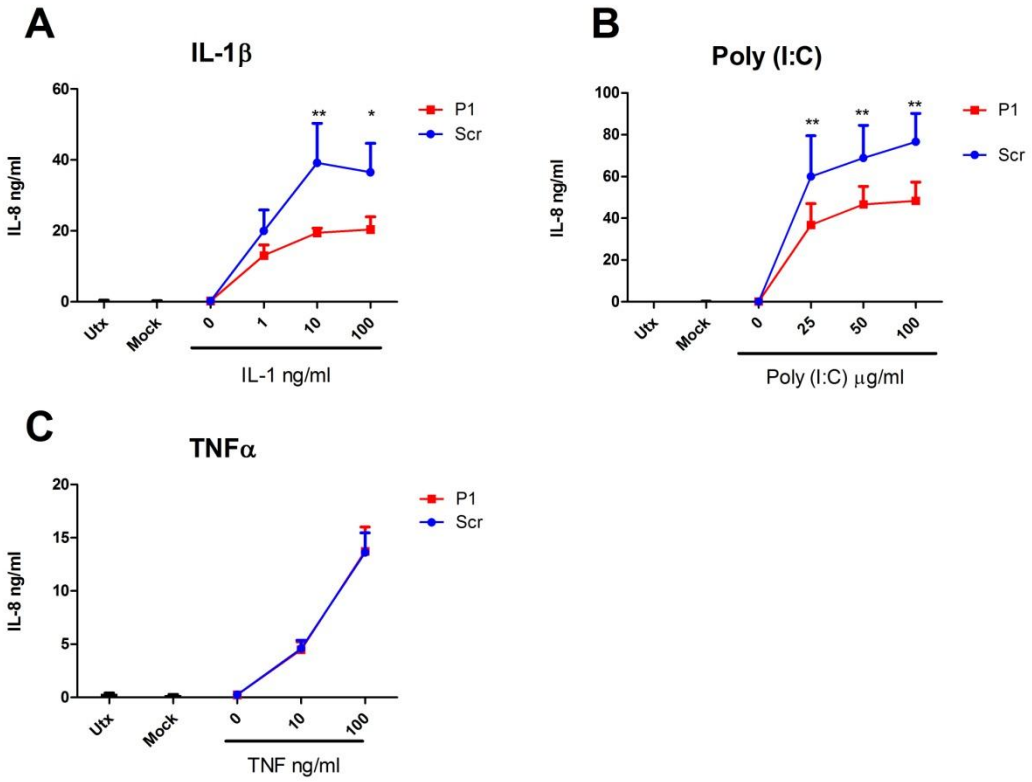


Figure 5.5: IL-8 generation in Pellino1 knockdown BEAS-2Bs in response to IL-1β, poly(I:C) and TNFα stimulation

BEAS-2Bs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of IL-1β (**A**), poly(I:C) (**B**), TNFα (**C**) or media (**A-C**). Untransfected (Utx) and mock transfected BEAS-2B cells were treated with media only. Supernatants were collected and analysed for IL-8 generation by ELISA. Data are expressed as mean±SEM from 5 independent experiments. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 for each concentration (*p<0.05, **p<0.01 and ***p<0.001).

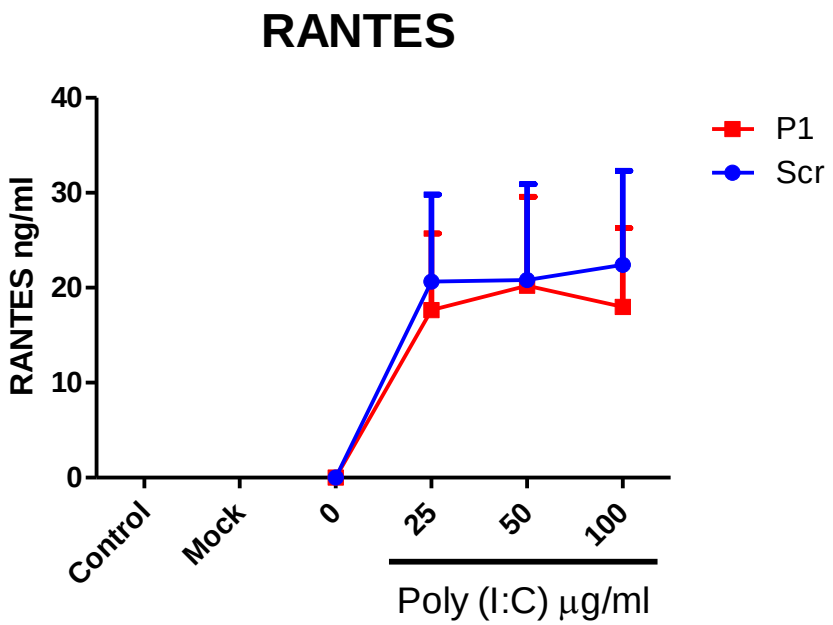


Figure 5.6: RANTES generation in Pellino1 knockdown BEAS-2Bs in response to poly(I:C) stimulation

BEAS-2Bs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of poly(I:C). Untransfected (Utx) and mock transfected BEAS-2B cells were treated with media only. Supernatants were collected and analysed for RANTES generation by ELISA. Data are expressed as mean±SEM from 3 independent experiments.

5.4 Effect of transient Pellino1 knockdown on interferon production in response to poly(I:C) in BEAS-2B.

Viral stimulus of cells leads to the activation of interferons (IFNs), which lead to the production of antiviral cytokines and inhibition of viral replication. Observed IL-8 responses to poly(I:C) were significantly reduced in Pellino1 knockdown BEAS-2B cells, however production of the ISG RANTES was preserved. This would assume that the IFNs would also be preserved in Pellino1 knockdown BEAS-2B cells. This was measured using specific qPCR primer-probes to measure INF-β (Type I), IFN-λ1 (Type III) and IFN-λ2/3 (Type III) production after 24 hours of poly(I:C) stimulation in Pellino1 knockdown and scrambled siRNA transfected BEAS-2B cells. Pellino1 knockdown had no effect on IFN-β (**Figure 5.7a**), IFN-λ1 (**Figure 5.7b**) or IFN-λ2/3 production (**Figure 5.7c**) in BEAS-2B cells in response to poly(I:C), which is in keeping with the preserved production of the ISG, RANTES in Pellino1 knockdown BEAS-2B cells (**Figure 5.6**).

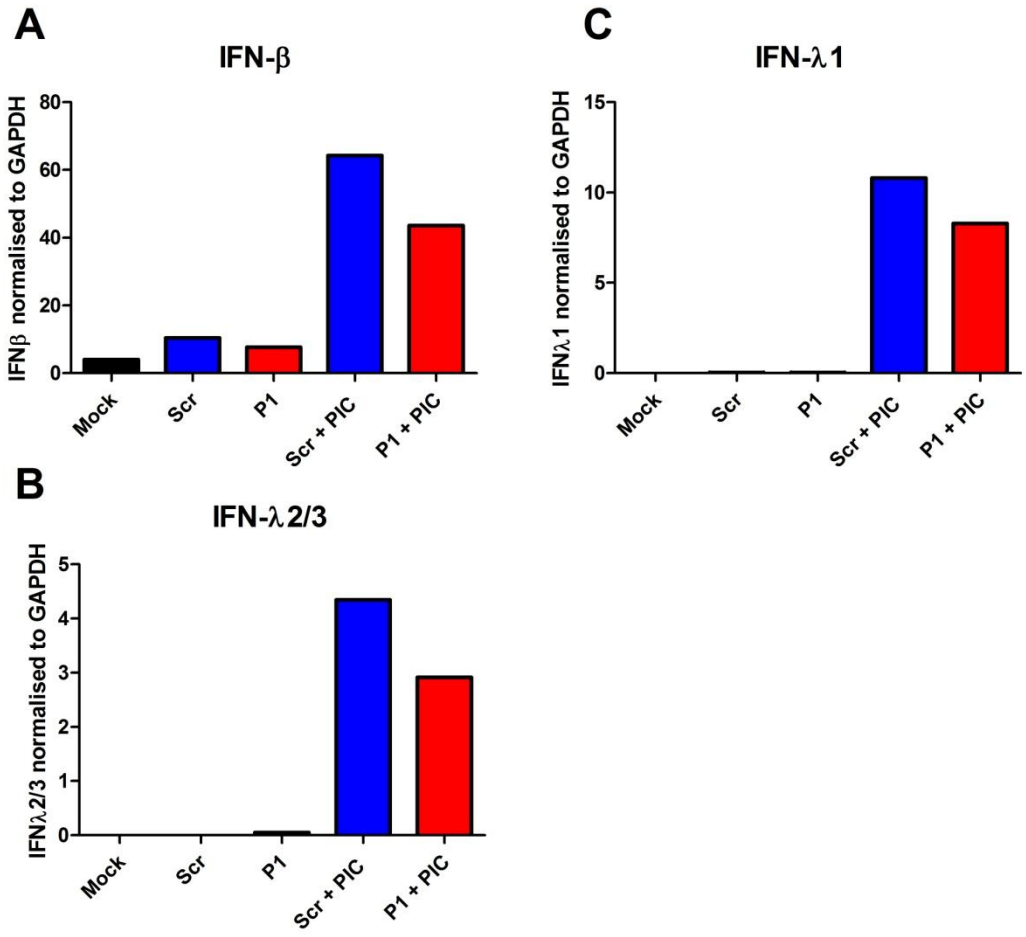


Figure 5.7: Interferon production in response to poly(I:C) in BEAS-2Bs

BEAS-2Bs were transiently transfected with siRNA targeting Pellino1 (P1) or a non-targeting scrambled siRNA (Scr) and were treated for 24 hours with a concentration response of poly(I:C) (PIC). Mock transfected BEAS-2Bs were treated with media only. Total RNA was extracted from these cells 48 hours post-transfection and converted into cDNA. Quantitative PCR qPCR was used to measure the interferon-β (IFNβ) (**A**), interferon-λ1 (IFNλ1) (**B**) and interferon-λ2/3 (IFNλ2/3) (**C**) mRNA expression levels, which were normalised to GAPDH as a loading control. Data are expressed as the mean from 2 independent experiments.

5.5 Effect of transient knockdown of Pellino1 on cytokine release in response to TLR/IL-1R agonists in primary bronchial epithelial cells

As BEAS-2Bs are a cell-line, they are not always representative of primary airway epithelium as cell signalling pathways that are not utilised in normal airway cells may be switched on in response to the immortalisation process. Thus, the physiological functional importance of Pellino1 in lung airway epithelium was investigated by transiently transfecting Pellino1 siRNA into human PBECs leading to target gene knockdown.

Pellino1 transcripts were significantly reduced in Pellino1 knockdown PBECs compared to control and scrambled siRNA transfected PBECs, which were measured using qPCR (**Figure 5.8**).

Interestingly, in contrast to published data that suggests Pellino1 is involved in the IL-1 signalling pathway (Jiang, Johnson et al. 2003) and previous work in the lung airway cell-line BEAS-2B, IL-8 generation in response to IL-1β was preserved in Pellino1 knockdown PBECs (**Figure 5.9a**). Strikingly however, transient Pellino1 knockdown in PBECs led to significant reduction in IL-8 generation in response to poly(I:C) (**Figure 9b**) and this finding was even more profound than in the BEAS-2B cell-line (**Figure 5.5b**). As expected, Pellino1 knockdown had no effect on TNFα-induced IL-8 production (**Figure 5.9c**).

IL-6 generation, which was measured as another classically NF-κB-regulated cytokine, was not significantly affected in Pellino1 knockdown PBECs in response to both IL-1β, poly(I:C) or TNFα (**Figure 5.10a-c**). As in HeLa and BEAS-2B cells, RANTES production was unaffected by Pellino1 knockdown in PBECs (**Figure 5.11a**), however we wanted to investigate whether other ISGs were also preserved and found that IP-10 was reduced but only at the highest dose of Poly(I:C) (25 μg/ml) (**Figure 5.11b**).

As IL-8 was profoundly reduced in response to poly(I:C) in Pellino1 knockdown PBECs, production of a range of other cytokines was measured in Pellino1 knockdown or scrambled siRNA transfected PBECs that had been stimulated with poly(I:C) using a Cytometric Bead Array (CBA). The CBA was used to give rise to potential insights into the pathways that are regulated by Pellino1. It was found that several other proinflammatory cytokines were also reduced in Pellino1 knockdown PBECs stimulated with poly(I:C), including TNFα (90% reduction), GM-CSF (79% reduction) and MCP-1 (74% reduction) and to a lesser extent MIP-1α (45% reduction) and MIP-1β (56% reduction) (**Figure 5.12a-e)**.

Other cytokines including as IL-1β and IL-1α were unaffected by Pellino1 **(Figure 5.12g+h)**. The anti-inflammatory cytokine IL-10 was only detected in very small concentrations (approximately 1 pg/ml) in PBECs stimulated with poly(I:C) **(Figure 5.12f).**

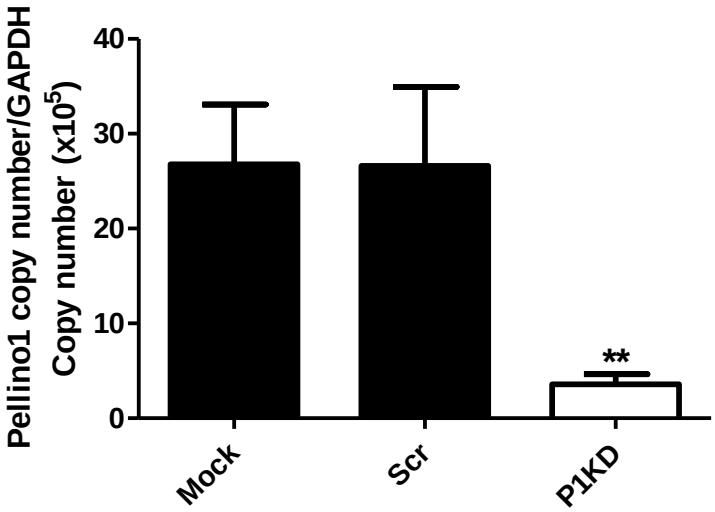


Figure 5.8: Transient Pellino1 knockdown at the mRNA level in primary bronchial epithelial cells

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA. Total RNA was extracted from these cells 48 hours post-transfection and converted into cDNA. Quantitative PCR was used to compare Pellino1 mRNA expression levels in mock and scrambled siRNA (Scr) transfected to that of Pellino1 knockdown PBECs. Pellino1 copy number was normalised to GAPDH copy number as a loading control and data are expressed as mean±SEM from 7 independent experiments from 4 different donors. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 (**$p<0.01$).

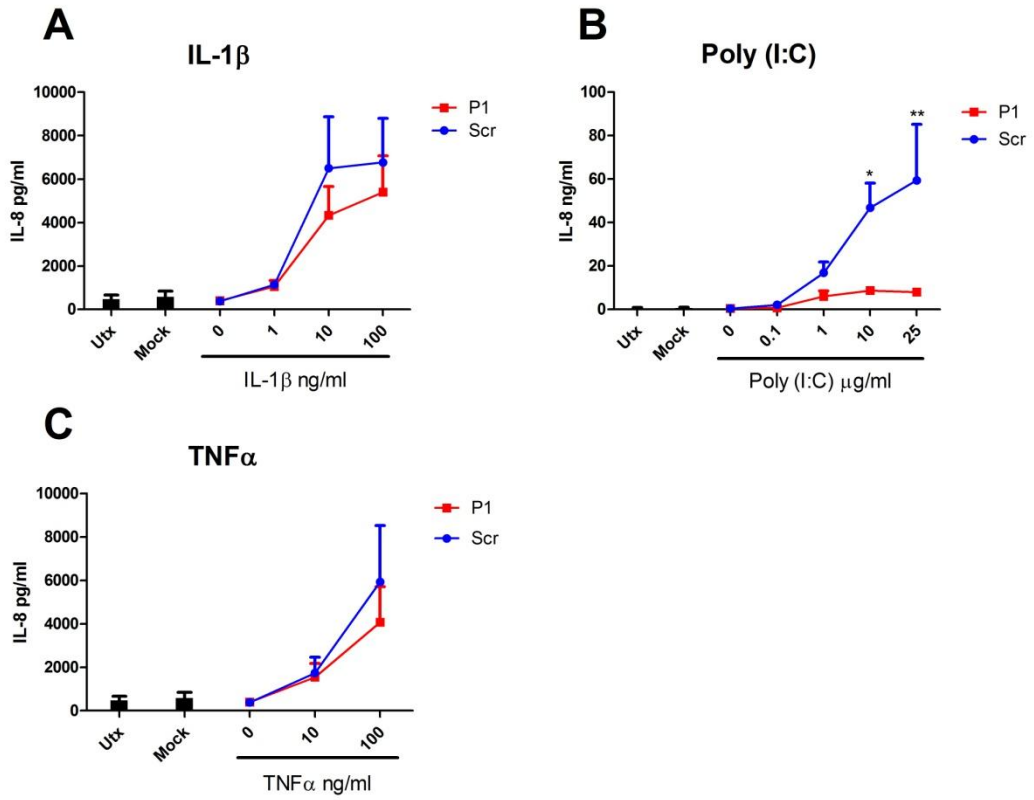


Figure 5.9: IL-8 generation in Pellino1 knockdown primary bronchial epithelial cells in response to IL-1β, poly(I:C) and TNFα stimulation

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of IL-1β (**A**), TNFα (**B**), poly(I:C) (**C**) or media (**A-C**). Untransfected (Utx) and mock transfected PBECs cells were treated with media only. Supernatants were collected and analysed for IL-8 generation by ELISA. Data are expressed as mean±SEM from 5 independent experiments. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 for each concentration (*p<0.05, **p<0.01 and ***p<0.001).

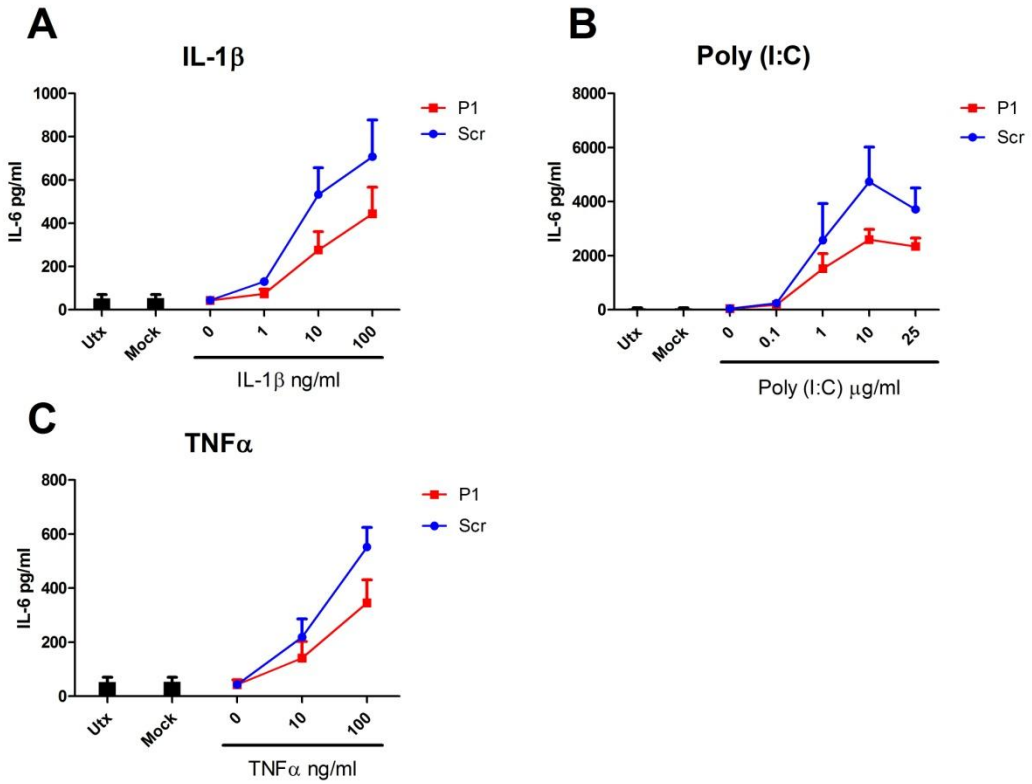


Figure 5.10: IL-6 generation in Pellino1 knockdown primary bronchial epithelial cells in response to IL-1β, poly(I:C) and TNFα stimulation

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of IL-1β (**A**), TNFα (**B**), poly(I:C) (**C**) or media (**A-C**). Untransfected (Utx) and mock transfected PBECs cells were treated with media only. Supernatants were collected and analysed for IL-6 generation by ELISA. Data are expressed as mean±SEM from 4 independent experiments. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 for each concentration (data did not reach statistical significant).

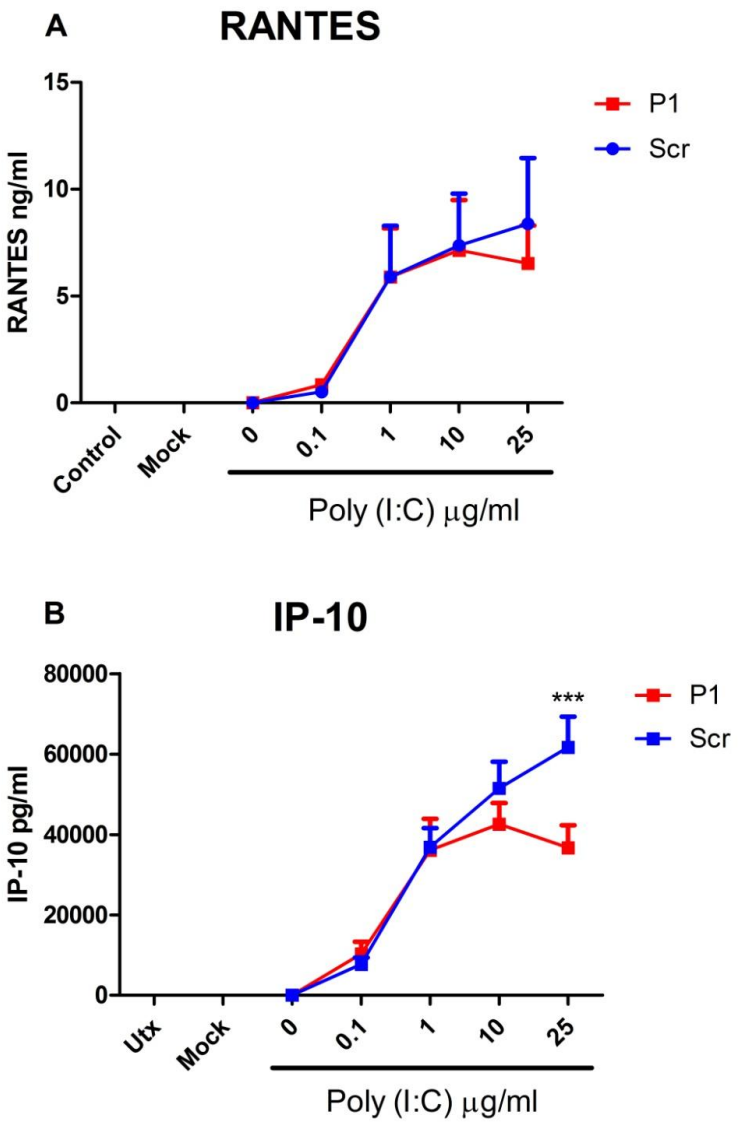


Figure 5.11: RANTES and IP-10 generation in Pellino1 knockdown primary bronchial epithelial cells in response to poly(I:C) stimulation

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of poly(I:C). Untransfected (Utx) and mock transfected PBECs were treated with media only. Supernatants were collected and analysed for RANTES (**A**) and IP-10 (**B**) generation by ELISA. Data are expressed as mean±SEM from 3 independent experiments. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 for each concentration (***$p<0.001$).

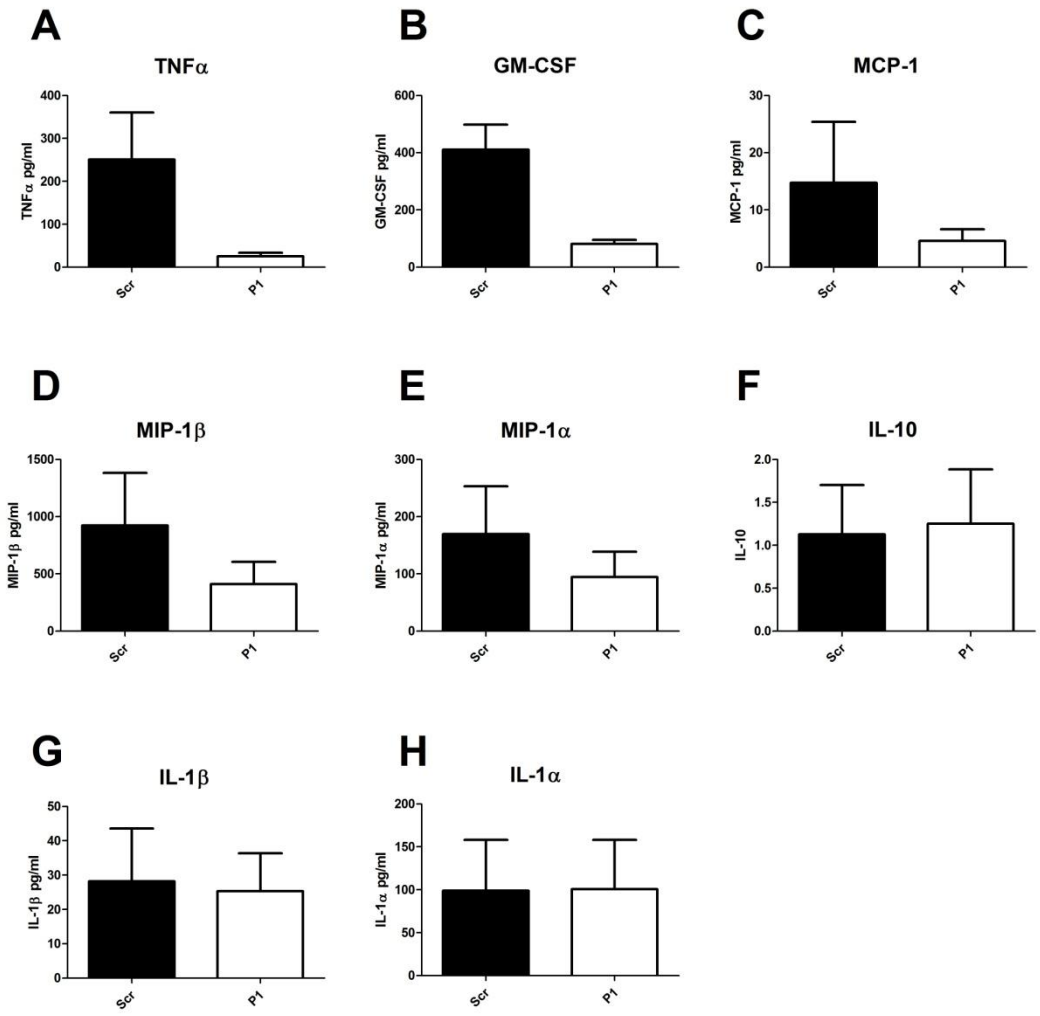


Figure 5.12: Cytokine release in response to poly(I:C) in primary bronchial epithelial cells measured by CBA

PBECs were transiently transfected with siRNA targeting Pellino1 (P1) or a non-targeting scrambled siRNA (Scr) and were treated for 24 hours with 25 µg/ml poly(I:C). Supernatants were collected and analysed for cytokine generation by cytokine bead array. Data are expressed as mean±SEM and supernatants were collected from 3 independent donors. Differences between Scr and P1 targeted cells did not reach statistical significance (Student's t test).

5.6 Effect of transient Pellino1 knockdown on interferon production in response to poly(I:C) in primary bronchial epithelial cells

Viral stimulus of cells leads to the activation of interferons (IFNs), which lead to the production of antiviral cytokines and inhibition of viral replication. The ISG RANTES was unaffected by Pellino1 knockdown in both BEAS-2B and PBECs and it would follow that IFN production was also unaffected, which was seen in BEAS-2B Pellino1 knockdown cells (**Figure 5.7**). However, unlike RANTES, another ISG IP-10 was significantly reduced in Pellino1 knockdown PBECs, but only at the highest dose of poly(I:C) (**Figure 5.11b**). Therefore we wanted to measure IFN production in Pellino1 knockdown PBECs to investigate further the potential selective role of Pellino1 in the regulation of viral signalling pathways.

The effect of Pellino1 knockdown on INF-β (type I), IFN-λ1 (type III) and IFN-λ2/3 (type III) transcription was investigated using specific qPCR primer-probes following poly(I:C) stimulation. Pellino1 knockdown led to a reduction (not significant) in IFN-β (**Figure 5.13a**), IFN-λ1 (**Figure 5.13b**) and IFN-λ2/3 production (**Figure 5.13c**) in response to poly(I:C) in PBECs.

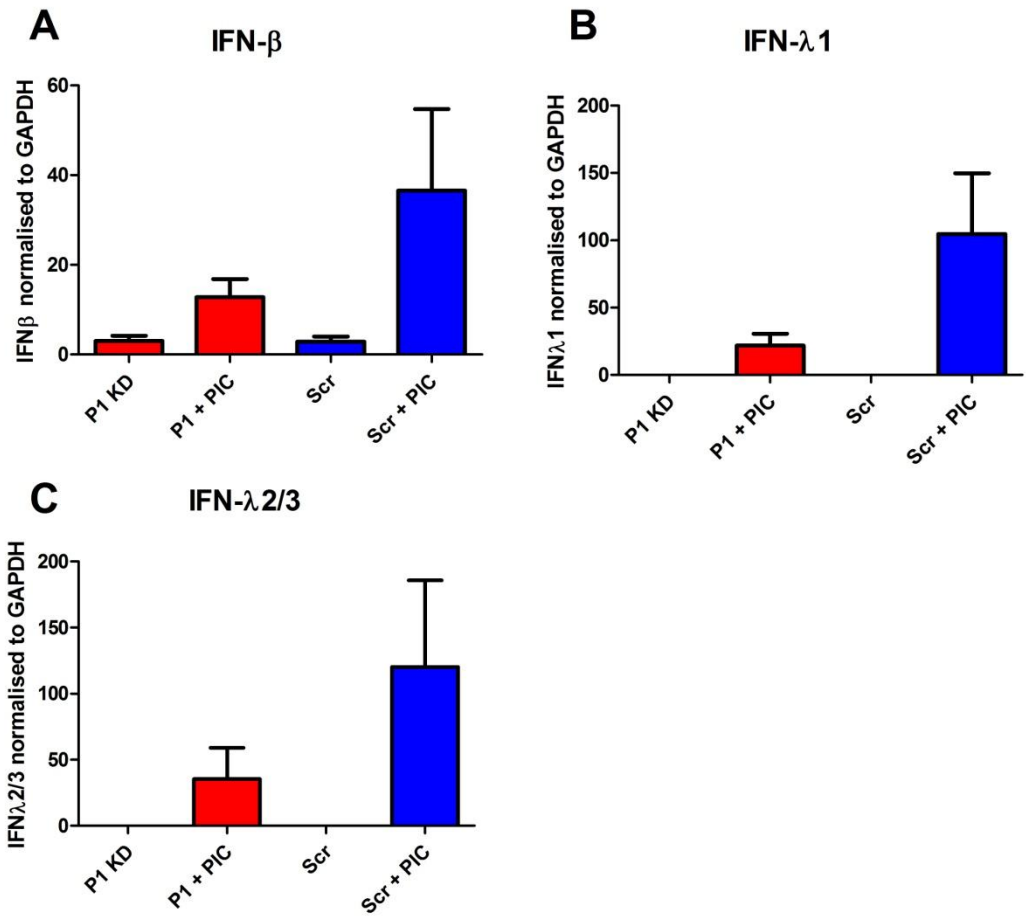


Figure 5.13: Interferon production in response to poly(I:C) in Pellino1 knockdown primary bronchial epithelial cells

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of poly(I:C). Total RNA was extracted from these cells 48 hours post-transfection and converted into cDNA. Quantitative PCR was used to measure the interferon-β (IFNβ) (**A**), interferon-λ1 (IFNλ1) (**B**) and interferon-λ2/3 (IFNλ2/3) (**C**) mRNA expression levels, which were normalised to GAPDH as a loading control. Data are expressed as mean±SEM from 3 independent experiments. Differences in IFN release between poly(I:C)-treated Scr and P1 cells did not reach statistical significance (Student's t test).

5.7 Effect of transient knockdown of RIP1 on cytokine release in response to TLR/IL-1R agonists in primary bronchial epithelial cells

During my thesis, a paper was published that suggested Pellino1 is involved in the regulation of the TLR3 signalling pathway and that this could be through the regulation of RIP1 (Chang, Jin et al. 2009). RIP1 is a death-domain kinase that was originally linked to TNFα signalling, where it interacts directly with TNF receptor 1 (TNFR1) to activate IKK thus leading to NF-κB and p38 MAPK activation (Hsu, Huang et al. 1996; Kelliher, Grimm et al. 1998). Upon recruitment to the TNFR1, RIP1 undergoes Lys-63 polyubiquitination by the E3 ubiquitin ligase TRAF2 (Hsu, Shu et al. 1996) which leads to TAK1 activation (Kanayama, Seth et al. 2004). More recently, RIP1 has been linked to TLR3/4 signalling where it is thought to bind to TRIF and facilitate in the activation of the IKK complex leading to NF-κB activation (Meylan, Burns et al. 2004; Cusson-Hermance, Khurana et al. 2005). Pellino1 has been suggested as the E3 ubiquitin ligase involved in TLR3-mediated Lys-63 polyubiquitination of RIP1 (Chang, Jin et al. 2009). These data were in keeping with data that had been gathered during my PhD that had shown that Pellino1 knockdown in airway epithelial cells leads to the significant reduction of IL-8 in response to poly(I:C). We hypothesised that if RIP1 was the Pellino1 target, then knockdown of RIP1 would recapitulate the Pellino1 knockdown phenotype.

To test this hypothesis, siRNA was used to knockdown RIP1 in PBECs which led to the reduction of RIP1 protein levels when compared to a structural protein, actin, which was used as a loading control (**Figure 5.14**). It was hypothesised that siRNA knockdown of RIP1 would result in the same phenotype as Pellino1 knockdown in PBECs, therefore leading to a profound reduction in IL-8 generation in response to poly(I:C) stimulation. As RIP1 is known to have roles in TNFα signalling, it was also hypothesised that RIP1 knockdown would perturb IL-8 and IL-6 generation in response to TNFα stimulation. As expected, RIP1 knockdown led to preserved IL-8 and IL-6 production in response to IL-1β (**Figure 5.15a**) and RANTES and IP-10 production in response to poly(I:C) was unaffected by RIP1 knockdown (**Figure 5.17a+b**). Surprisingly, IL-8 and IL-6 generation in response to TNFα stimulation was also unaffected by RIP1 knockdown in PBECs (**Figure 5.15c+5.16c**). Another finding that was surprising was, in contrast to Pellino1 knockdown, RIP1 knockdown led to a significant increase in IL-8 generation in response to poly(I:C) in PBECs (**Figure 5.15b**), however IL-6 generation was only slightly increased at the highest dose of Poly(I:C) (25 μg/ml) (**Figure 5.16b**).

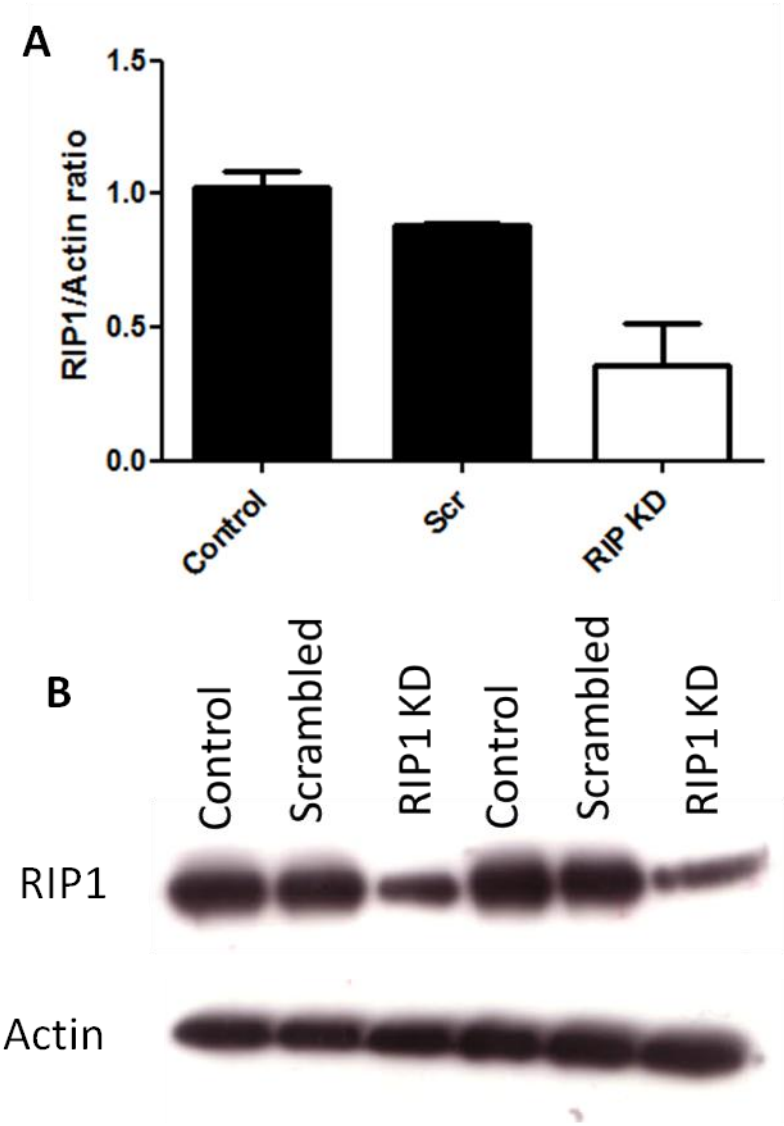


Figure 5.14: Transient RIP1 knockdown at the protein level in primary bronchial epithelial cells

PBECs were transiently transfected with siRNA targeting RIP1 or a non-targeting scrambled siRNA and cells were lysed and immunoblotted using antibodies to either RIP1 or actin (loading control). Densitometry was carried out and processed using NIH Image 1.62 analysis software. Data are expressed as mean±SEM from 3 independent experiments from a single donor (**A**). Representative immunoblot showing RIP1 knockdown (**B**). Statistical analysis carried out on densitometry by one-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 (data did not reach statistical significant).

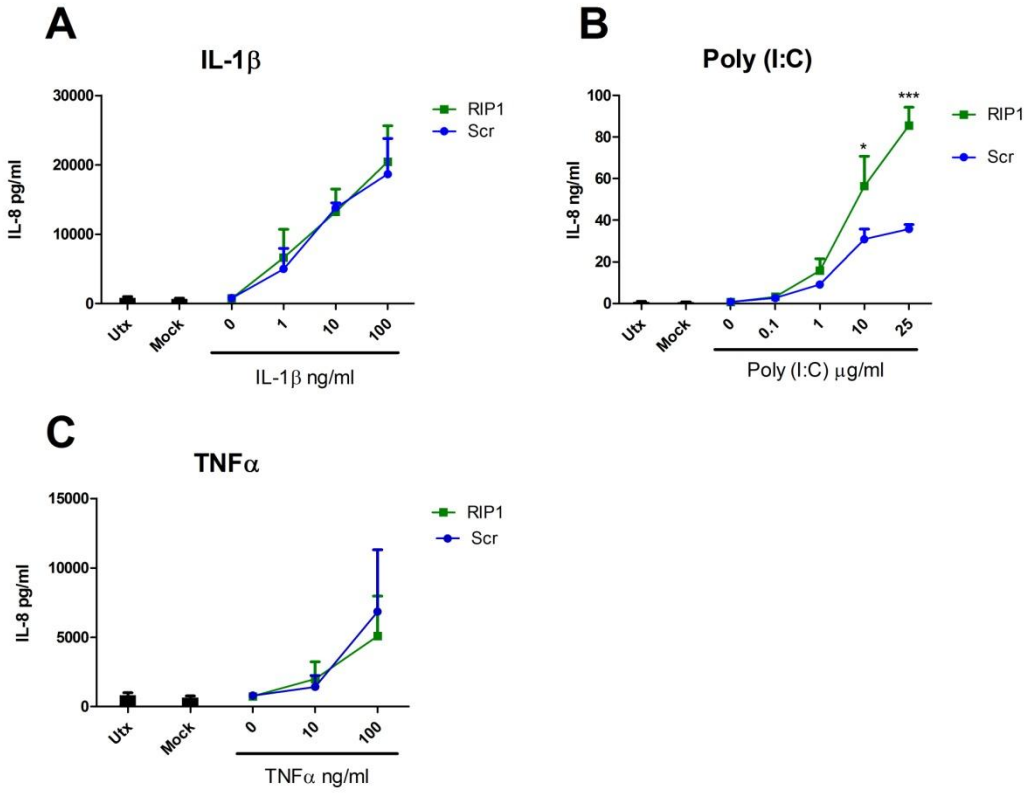


Figure 5.15: IL-8 generation in RIP1 knockdown primary bronchial epithelial cells in response to IL-1β, poly(I:C) and TNFα stimulation

PBECs were transiently transfected with siRNA targeting RIP1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of IL-1β (**A**), poly(I:C) (**B**), TNFα (**C**) or media (**A-C**). Untransfected (Utx) and mock transfected PBECs cells were treated with media only. Supernatants were collected and analysed for IL-8 generation by ELISA. Data are expressed as mean±SEM from 3 independent experiments. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 for each concentration (*p<0.05, ***p<0.001).

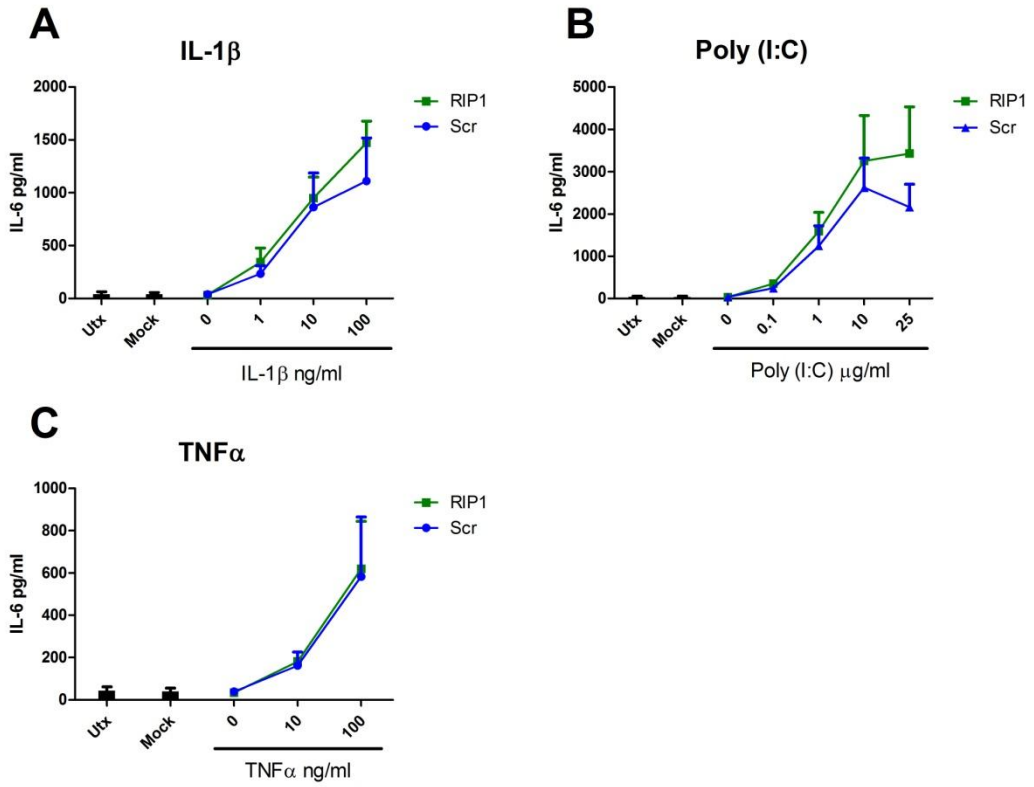


Figure 5.16: IL-6 generation in RIP1 knockdown primary bronchial epithelial cells in response to IL-1β, poly(I:C) and TNFα stimulation

PBECs were transiently transfected with siRNA targeting RIP1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of IL-1β (**A**), poly(I:C) (**B**), TNFα (**C**) or media (**A-C**). Untransfected (Utx) and mock transfected PBECs cells were treated with media only. Supernatants were collected and analysed for IL-6 generation by ELISA. Data are expressed as mean±SEM from 3 independent experiments. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 for each concentration (data not significant).

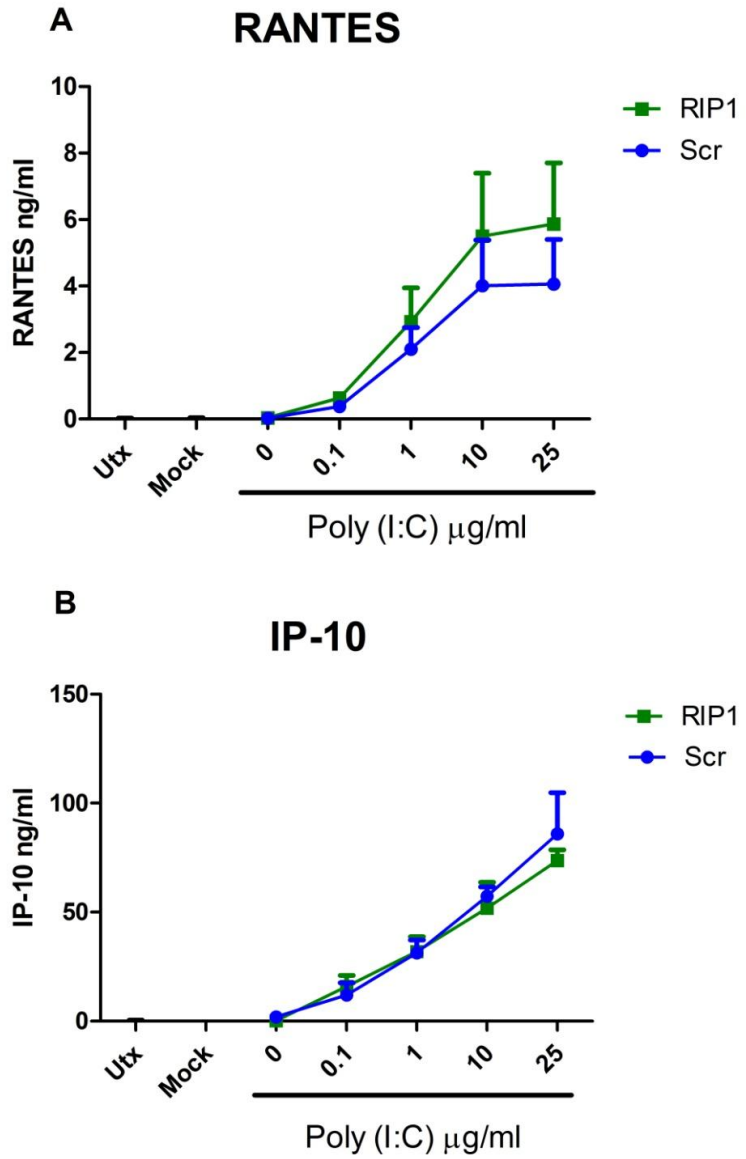


Figure 5.17: RANTES and IP-10 generation in RIP1 knockdown primary bronchial epithelial cells in response to poly(I:C) stimulation

PBECs were transiently transfected with siRNA targeting RIP1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of poly(I:C). Untransfected (Utx) and mock transfected PBECs were treated with media only. Supernatants were collected and analysed for RANTES (**A**) and IP-10 (**B**) generation by ELISA. Data are expressed as mean±SEM from 3 independent experiments. Statistical analysis carried out by one-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 for each concentration (data not significant).

5.8 Effect of transient knockdown of Pellino1 on cytokine release in response to rhinovirus in BEAS-2B

Previous data suggested a role for Pellino1 in the TLR3 signalling pathway in airway epithelial cells, as Pellino1 knockdown in both BEAS-2B cells and PBECs led to the significant reduction in IL-8 production in response to poly(I:C). We then wanted to investigate the role of Pellino1 in response to a natural human viral pathogen as, unlike with poly(I:C) stimulation, infection with a natural virus would lead to the physiological activation of receptors and viral signalling pathways that may not occur in the absence of replicating virus. To investigate the role of Pellino1 in viral signalling pathways in airway epithelial cells, Pellino1 knockdown BEAS-2B were infected with rhinovirus 1B (RV1B).

Rhinoviruses are small (25-30 nm diameter), non-enveloped viruses belonging to the *Picornaviridae* family of viruses that infect the upper respiratory tract. Rhinoviral infections have been shown to be a major cause of asthma exacerbations (Atmar, Guy et al. 1998; Grissell, Powell et al. 2005). RV1B belongs to the minor group of rhinoviruses and so enters host airway epithelial cells by binding to very low-density lipoprotein receptor (vLDLR) on the cell surface (Marlovits, Abrahamsberg et al. 1998; Marlovits, Zechmeister et al. 1998).

To investigate the effect of Pellino1 knockdown on RV1B induced cytokine production, Pellino1 knockdown BEAS-2B cells were infected with a range of $TCID_{50}$ of RV1B for 1 hour and IL-8 and RANTES release 24 hours post-infection was measured using ELISA. Filtrate and UV-treated RV1B controls were included to ensure cytokine release was infection-dependent and not due to supernatant transfer from viral manufacturing or replication-deficient viral particles, respectively.

Pellino1 knockdown in lung airway epithelial cell-line BEAS-2B led to a significant reduction in IL-8 generation in response to the TLR3 agonist and viral mimic poly(I:C). To investigate whether this phenotype is apparent in response to a natural pathogen, Pellino1 knockdown BEAS-2B cells were infected with RV1B. Pellino1 transcript levels were significantly reduced in cells transfected with siRNA to Pellino 1 (**Figure 5.18**). Unexpectedly, infection of Pellino1 knockdown BEAS-2B with RV1B had no effect on IL-8 generation (**Figure 5.19**) or RANTES production (**Figure 5.20**).

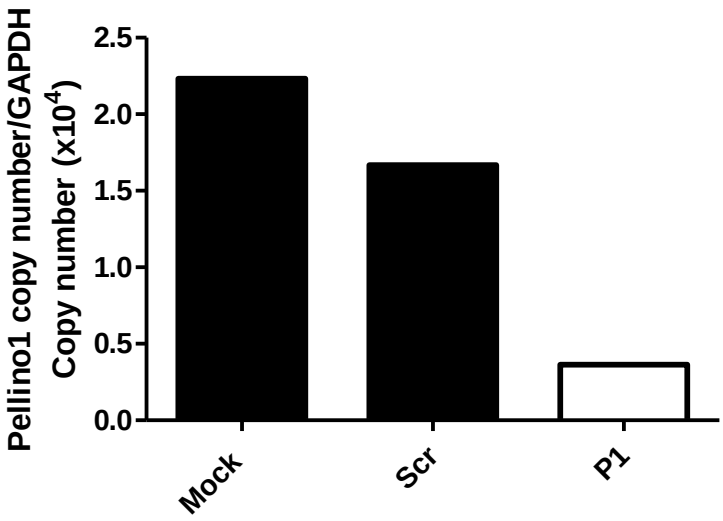


Figure 5.18: Transient Pellino1 knockdown at the mRNA level in BEAS-2Bs in response to rhinovirus infection

BEAS-2Bs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were infected with a range of $TCID_{50}$ of rhinovirus1B (RV1B) for 1 hour. Total RNA was extracted from these cells 48 hours post-transfection and converted into cDNA. Quantitative RT-PCR (qPCR) was used to compare Pellino1 mRNA expression levels in Mock and scrambled siRNA (Scr) transfected to that of Pellino1 knockdown PBECs. Pellino1 copy number was normalised to GAPDH copy number as a loading control and data is from a single experiment.

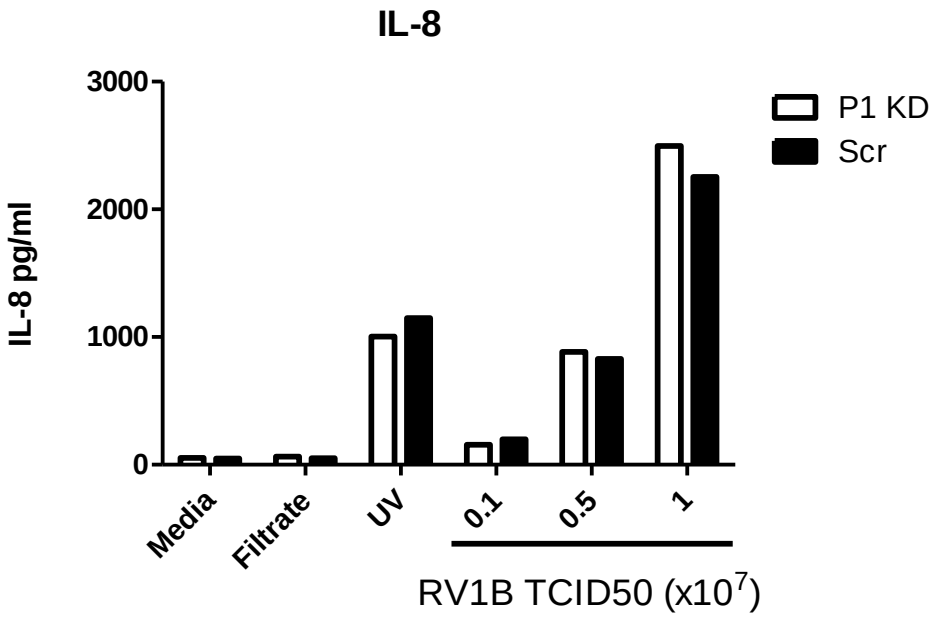


Figure 5.19: IL-8 generation in Pellino1 knockdown BEAS-2Bs in response to rhinovirus infection

BEAS-2Bs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were infected with a range of $TCID_{50}$ of rhinovirus1B (RV1B) for 1 hour. Media controls were treated with complete BEAS-2B medium, filtrate controls were treated with viral media that did not contain viral particles and UV controls were incubated with replication-deficient RV1B. Complete BEAS-2B medium replaced viral medium and supernatants were collected 24 hours later. These supernatants were analysed for IL-8 generation by ELISA and data are expressed as pg/ml IL-8 from a single experiment.

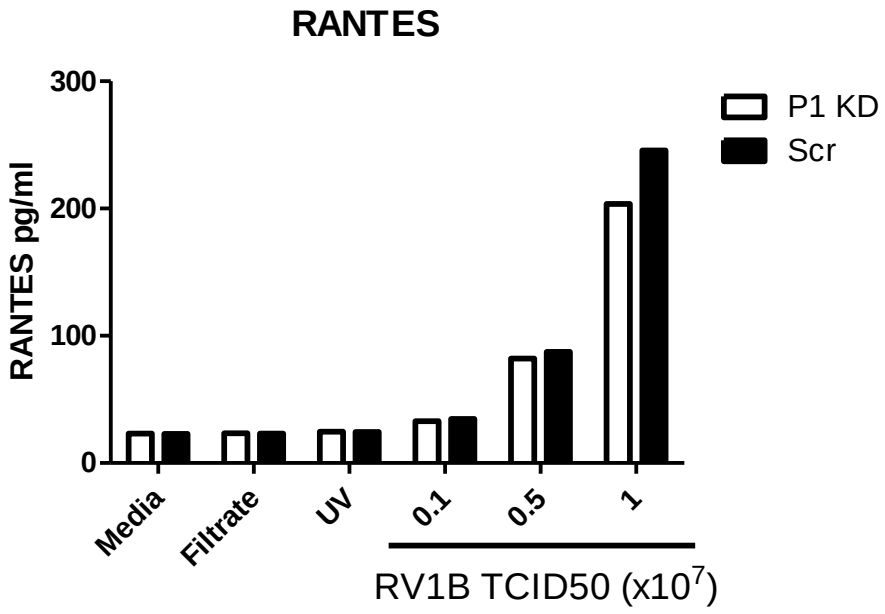


Figure 5.20: RANTES generation in Pellino1 knockdown BEAS-2Bs in response to rhinovirus infection

BEAS-2Bs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were infected with a range of $TCID_{50}$ of rhinovirus1B (RV1B) for 1 hour. Media controls were treated with complete BEAS-2B medium, filtrate controls were treated with viral media that did not contain viral particles and UV controls were incubated with replication-deficient RV1B. Complete BEAS-2B medium replaced viral medium and supernatants were collected 24 hours later. These supernatants were analysed for RANTES generation by ELISA and data are expressed as pg/ml RANTES from a single experiment.

5.9 Effect of transient knockdown of Pellino1 on cytokine release in response to rhinovirus in primary bronchial epithelial cells

Pellino1 knockdown in BEAS-2B cells had no effect on RV1B induced cytokine release despite these cells showing a reduction in IL-8 generation in response to the TLR3 agonist and viral mimic Poly(I:C). However, this phenotype is more profound in Pellino1 knockdown PBECs, where IL-8 release in response to 25 μg/ml poly(I:C) was reduced by 86.62% in Pellino1 knockdown PBECs (**Figure 5.9b**) in contrast to only 38.77% in Pellino1 knockdown BEAS-2B cells (**Figure 5.5b**). Therefore we wanted to investigate the role of Pellino1 in the more physiologically relevant cell-type, PBECs, in response to the natural viral pathogen, RV1B.

To investigate the effect of Pellino1 knockdown on RV1B induced cytokine production, Pellino1 knockdown PBECs were infected with a range of $TCID_{50}$ of RV1B for 1 hour and IL-8, IL-6, IP-10 and RANTES release 24 hours post-infection was measured using ELISA. Filtrate and UV-treated RV1B controls were also included and did not produce significant cytokine generation (data not shown). Data shown is from a single donor but are representative of 3 independent donors (data not shown). Pellino1 knockdown PBECs infected with RV1B showed a significant reduction in IL-8 (**Figure 5.21**) and IL-6 (**Figure 5.22**) generation, however RANTES (**Figure 5.23**) and IP-10 (**Figure 5.24**) production was unaffected by Pellino1 knockdown.

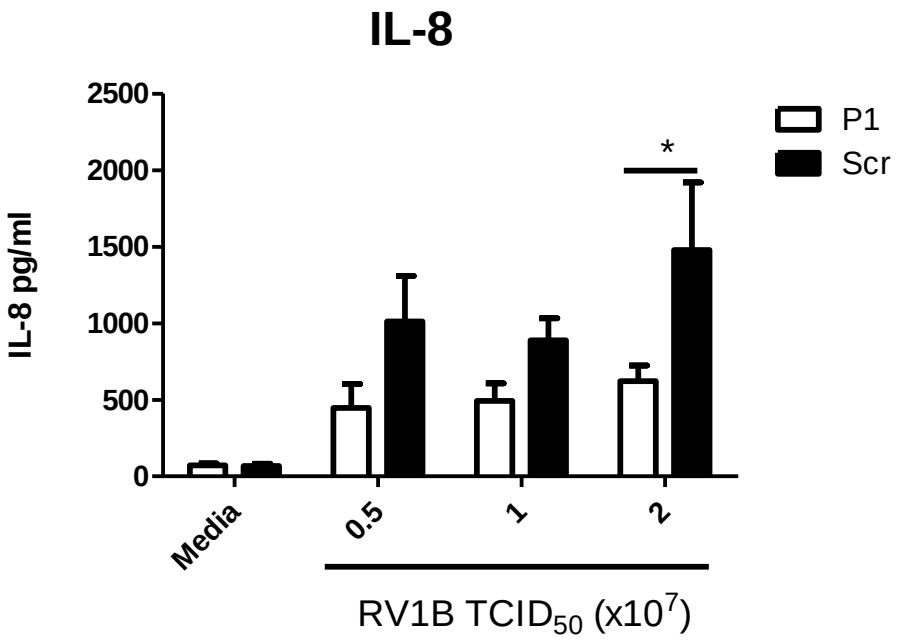


Figure 5.21: IL-8 generation in Pellino1 knockdown primary bronchial epithelial in response to rhinovirus infection

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were infected with a range of $TCID_{50}$ of rhinovirus1B (RV1B) for 1 hour. Media controls were treated with basal PBEC medium, filtrate controls were treated with viral media that did not contain viral particles and UV controls were incubated with replication-deficient RV1B (filtrate and UV data not shown). Basal PBEC medium replaced viral medium and supernatants were collected 24 hours later. These supernatants were analysed for IL-8 generation by ELISA and data are expressed as mean±SEM from 3 independent experiments from a single donor. Data are representative of 3 different donors (data not shown). Filtrate and UV controls did not produce cytokine above that of basal levels (data not shown). Statistical analysis carried out by two-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 (*$p<0.05$).

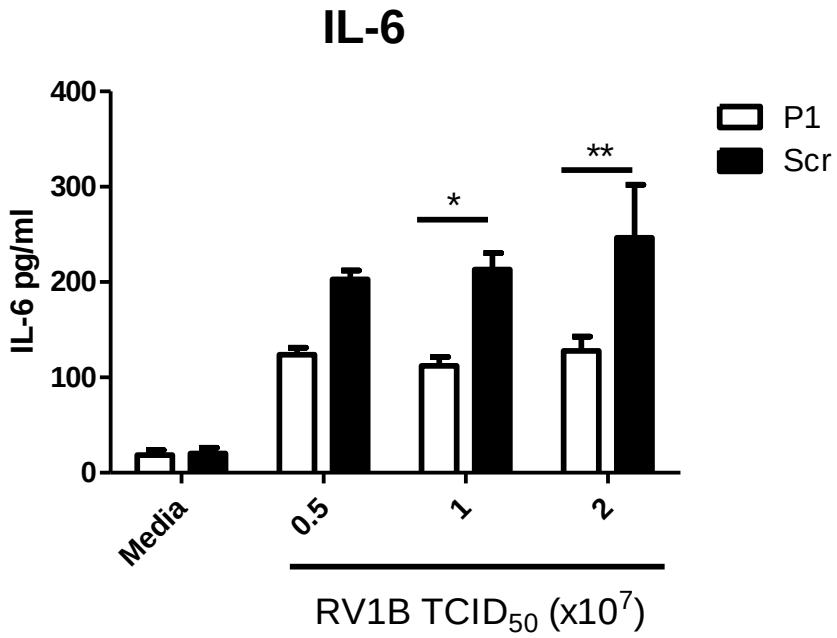


Figure 5.22: IL-6 generation in Pellino1 knockdown primary bronchial epithelial cells in response to rhinovirus infection

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were infected with a range of $TCID_{50}$ of rhinovirus1B (RV1B) for 1 hour. Media controls were treated with basal PBEC medium, filtrate controls were treated with viral media that did not contain viral particles and UV controls were incubated with replication-deficient RV1B (filtrate and UV data not shown). Basal PBEC medium replaced viral medium and supernatants were collected 24 hours later. These supernatants were analysed for IL-6 generation by ELISA and data are expressed as mean±SEM from 3 independent experiments from a single donor. Data are representative of 3 different donors (data not shown). Filtrate and UV controls did not produce cytokine above that of basal levels (data not shown). Statistical analysis carried out by two-way ANOVA with Bonferroni's post-test, comparing Scr vs. P1 (*$p<0.05$ and **$p<0.01$).

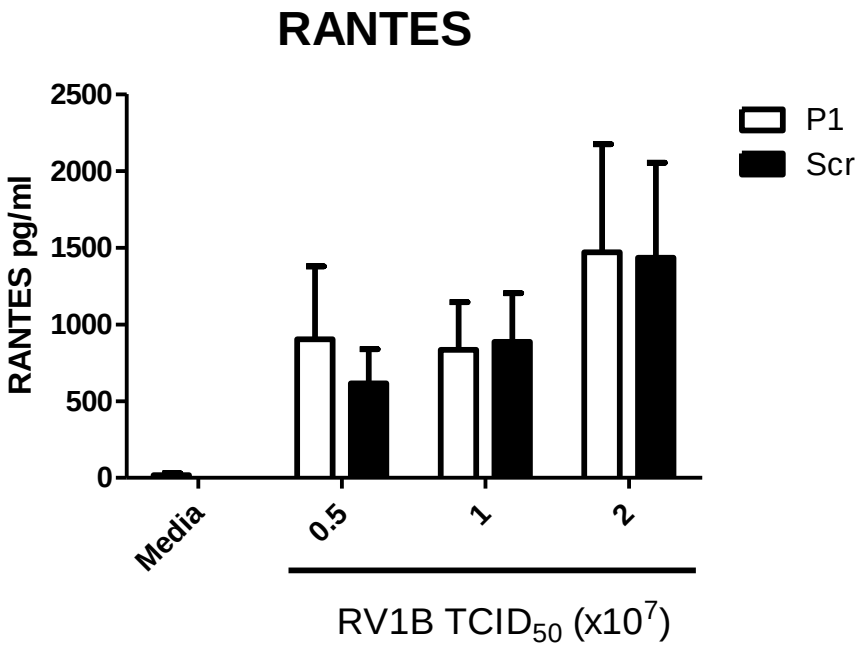


Figure 5.23: RANTES generation in Pellino1 knockdown primary bronchial epithelial cells in response to rhinovirus infection

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were infected with a range of $TCID_{50}$ of rhinovirus1B (RV1B) for 1 hour. Media controls were treated with basal PBEC medium, filtrate controls were treated with viral media that did not contain viral particles and UV controls were incubated with replication-deficient RV1B. Basal PBEC medium replaced viral medium and supernatants were collected 24 hours later. These supernatants were analysed for RANTES generation by ELISA and data are expressed as mean±SEM from 3 independent experiments from a single donor. Data are representative of 3 different donors (data not shown). Filtrate and UV controls did not produce cytokine above that of basal levels (data not shown).

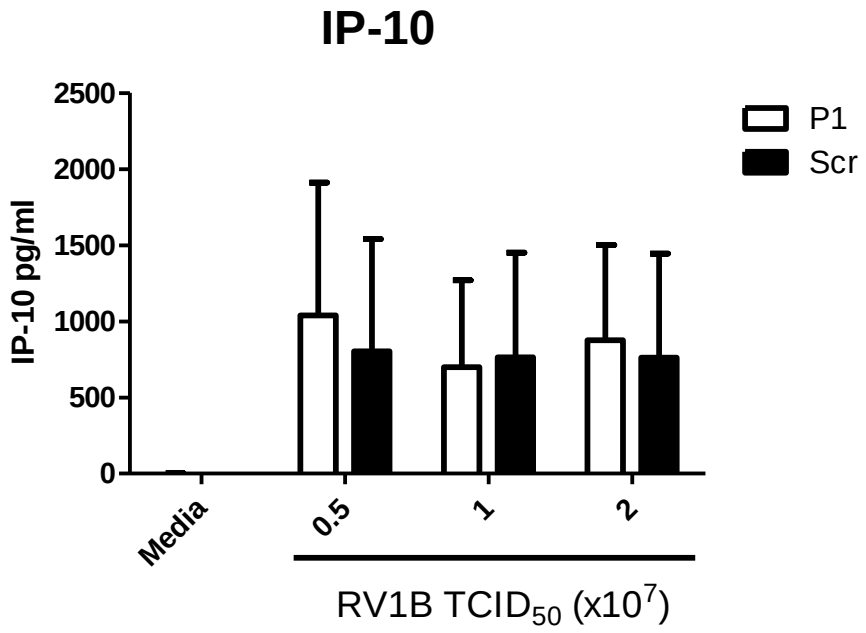


Figure 5.24: IP-10 generation in Pellino1 knockdown primary bronchial epithelial cells in response to rhinovirus infection

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were infected with a range of $TCID_{50}$ of rhinovirus1B (RV1B) for 1 hour. Media controls were treated with basal PBEC medium, filtrate controls were treated with viral media that did not contain viral particles and UV controls were incubated with replication-deficient RV1B. Basal PBEC medium replaced viral medium and supernatants were collected 24 hours later. These supernatants were analysed for IP-10 generation by ELISA and data are expressed as mean±SEM from 3 independent experiments from a single donor. Data are representative of 3 different donors (data not shown). Filtrate and UV controls did not produce cytokine above that of basal levels (data not shown).

5.10 Effect of transient Pellino1 knockdown on interferon production in response to rhinovirus in BEAS-2B and primary bronchial epithelial cells

Rhinoviruses infect epithelial cells of the upper respiratory tract, which leads to the induction of type I IFNs (IFN-β) and type III (IFN-λ) IFNs (Khaitov, Laza-Stanca et al. 2009). To investigate the effect of Pellino1 knockdown on RV1B induced IFN production, Pellino1 knockdown BEAS-2B cells or PBECs were infected with RV1B using a $TCID_{50}$ of $2X10^7$ for 1 hour and IFN release 24 hours post-infection was measured using qPCR.

The NF-κB-regulated cytokines IL-8 and IL-6 were reduced in Pellino1 knockdown PBECs in response to RV1B (**Figures 5.21 + 5.22**), however RANTES and IP-10 were unaffected by Pellino1 knockdown (**Figures 5.23 + 5.24**). Following these findings, it was hypothesised that IFN production in lung airway epithelial would be unaffected by Pellino1 knockdown. This was investigated using qPCR to measure Type I and III IFN production in BEAS-2B cells and PBECs 24 hours post-RV1B infection. Pellino1 knockdown was found to have no significant effect on RV1B induced IFN-β (**Figure 5.25a**), IFN-λ1 (**Figure 5.25b**) or IFN-λ2/3 (**Figure 5.25c**) in PBECs. RV1B infection of Pellino1 knockdown BEAS-2B cells was also found to have no significant effect on RV1B induced IFN-β (**Figure 5.26a**), IFN-λ1 (**Figure 5.26b**) or IFN-λ2/3 (**Figure 5.26c**).

5.11 Effect of transient Pellino1 knockdown on viral replication in primary bronchial epithelial cells

Interferons have been shown to inhibit rhinoviral replication as a way to contribute to the control of the infection (Cakebread, Xu et al. 2011). As Pellino1 knockdown has no effect on IFN release in RV1B infected PBECs, it was hypothesised that replication of the RV1B would also be unaffected by Pellino1 knockdown.

To test the effect of Pellino1 knockdown on viral replication, Pellino1 knockdown PBECs were infected with a $TCID_{50}$ of $2.0x10^7$ of RV1B for 1 hour and 24 hours post-infection viral copies were measured using qPCR.

Pellino1 knockdown had no significant effect on RV1B replication in PBECs (**Figure 5.27**), which is interesting as this contrasts with previous work from our group that shows that knockdown of another key regulator of the TLR/IL-1R signalling pathway, MyD88, in airway epithelial cells leads to the significant increase in RV1B replication (Stokes, Ismail et al. 2011).

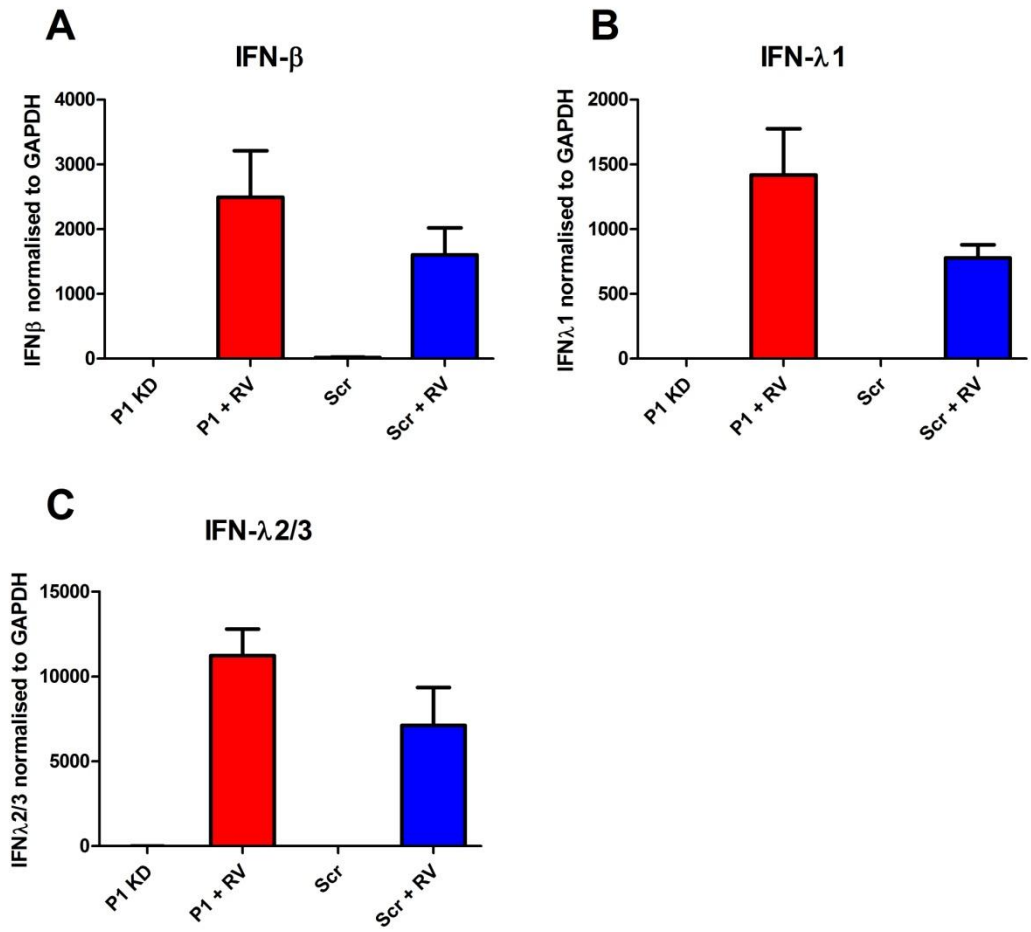


Figure 5.25: Interferon production in response to rhinovirus in Pellino1 knockdown primary bronchial epithelial cells

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 1 hour with a $TCID_{50}$ of $2X10^7$ of rhinovirus1B (RV1B). Media controls were treated with basal PBEC medium. Total RNA was extracted from these cells 48 hours post-transfection and converted into cDNA. Quantitative PCR was used to measure the interferon-β (IFNβ) (**A**), interferon-λ1 (IFNλ1) (**B**) and interferon-λ2/3 (IFNλ2/3) (**C**) mRNA expression levels, which were normalised to GAPDH as a loading control. Data are expressed as mean±SEM from 3 independent experiments from a single donor. Differences in IFN release between RV1B infected Scr and P1 cells did not reach statistical significance (Student's t test).

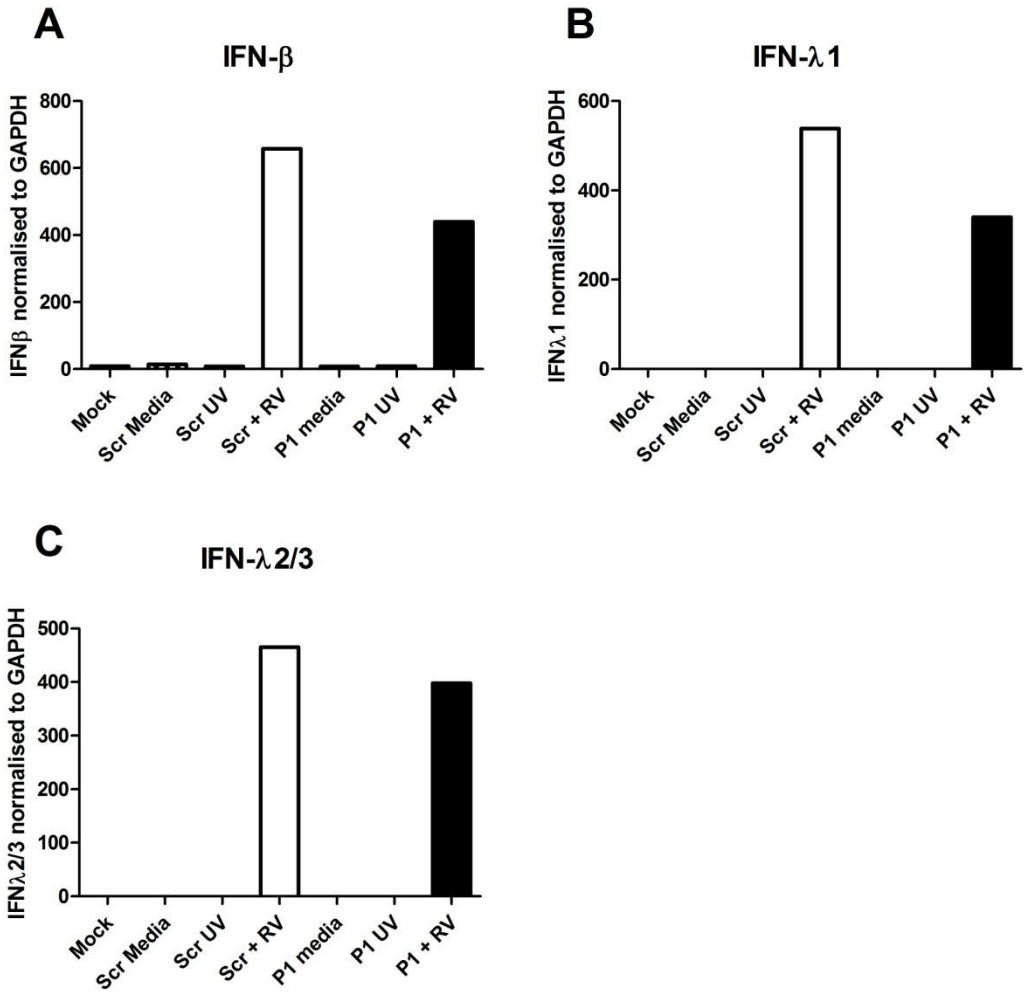


Figure 5.26: Interferon production in response to rhinovirus in Pellino1 knockdown BEAS-2Bs

BEAS-2Bs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 1 hour with a $TCID_{50}$ of $2X10^7$ of rhinovirus1B (RV1B). Mock and media controls were treated with complete BEAS-2B medium and UV controls were incubated with replication-deficient RV1B. Total RNA was extracted from these cells 48 hours post-transfection and converted into cDNA. Quantitative RT-PCR (qPCR) was used to measure the interferon-β (IFNβ) (**A**), interferon-λ1 (IFNλ1) (**B**) and interferon-λ2/3 (IFNλ2/3) (**C**) mRNA expression levels, which were normalised to GAPDH as a loading control. Data are from a single experiment.

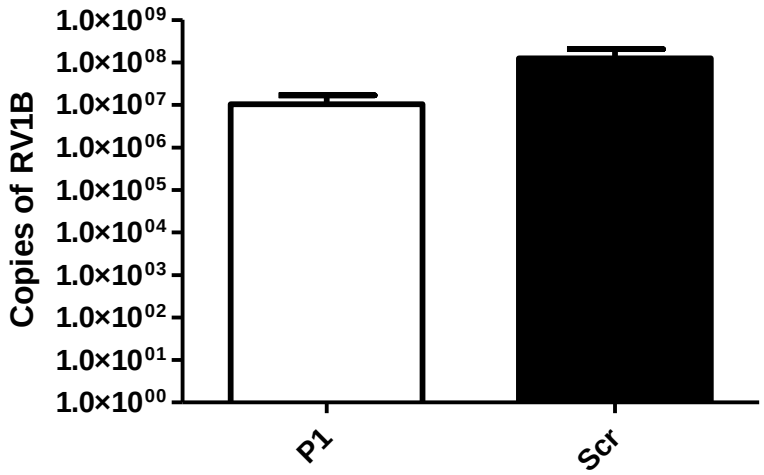


Figure 5.27: Viral replication in Pellino1 knockdown primary bronchial epithelial cells

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 1 hour with a $TCID_{50}$ of $2X10^7$ of rhinovirus1B (RV1B). Total RNA was extracted from these cells 48 hours post-transfection and converted into cDNA. Quantitative PCR was used to measure viral DNA copies and data are expressed on a Log10 scale as mean±SEM from 4 independent experiments from 2 different donors. Differences in RV1B copy number between Scr and P1 cells did not reach statistical significance (Student's t test).

5.12 Investigating antiviral signalling pathways in airway epithelial cells

Pellino1 knockdown led to the significant reduction in IL-8 in response to poly(I:C) in BEAS-2B cells and this phenotype was even more pronounced in Pellino1 knockdown PBECs. We wanted to investigate the contribution of NF-κB activation to pathways activated by poly(I:C).

5.12.1 Poly(I:C) induced IκB-α degradation in BEAS-2B

TLR and IL-1R signalling, with the exception of TLR3, is initiated through the activation of the MyD88 adaptor molecule, which leads to downstream signalling through activation of IRAK molecules (Akira and Sato 2003), TRAF6 (Yamaguchi, Shirakabe et al. 1995)and TAK1 (Takaesu, Kishida et al. 2000) which leads to the activation of the IKK complex and consequential phosphorylation and ubiquitination of the NF-κB-inhibitory proteins (IκBs) and targets them for degradation (Muzio, Polentarutti et al. 2000; Chen 2005). Thus, one way in which NF-κB activation is measured is by IκBα protein degradation. IL-1β activates the IL-1R and leads to NF-κB activation via the signalling molecules discussed above, however the viral mimic poly(I:C) activates MyD88-independent TLR3 signalling pathway that utilises the adaptor molecule TRIF and activates NF-κB via the recruitment of RIP1 which then in turn activates TAK1 (Han, Su et al. 2004; Meylan, Burns et al. 2004).

TLR3 signalling leads to both the activation of IRF3 and NF-κB, whereby activation of IRF3 is initiated by the activation of IKKε/TBK by TRIF and NF-κB is activated by TRIF activating RIP1 which leads to the activation of TAK1 and downstream mediators discussed above. As poly(I:C) activates TLR3 it was hypothesised that stimulation of BEAS-2B cells with poly(I:C) would lead to IκBα protein degradation with the same kinetics as IL-1β stimulation.

Stimulation of BEAS-2B cells with IL-1β or poly(I:C) led to IκBα degradation. Degradation of IκBα could be seen after 10 minutes of IL-1β stimulation (**Figure 5.28a**) and after 45 minutes with poly(I:C) stimulation (**Figure 5.28b**).

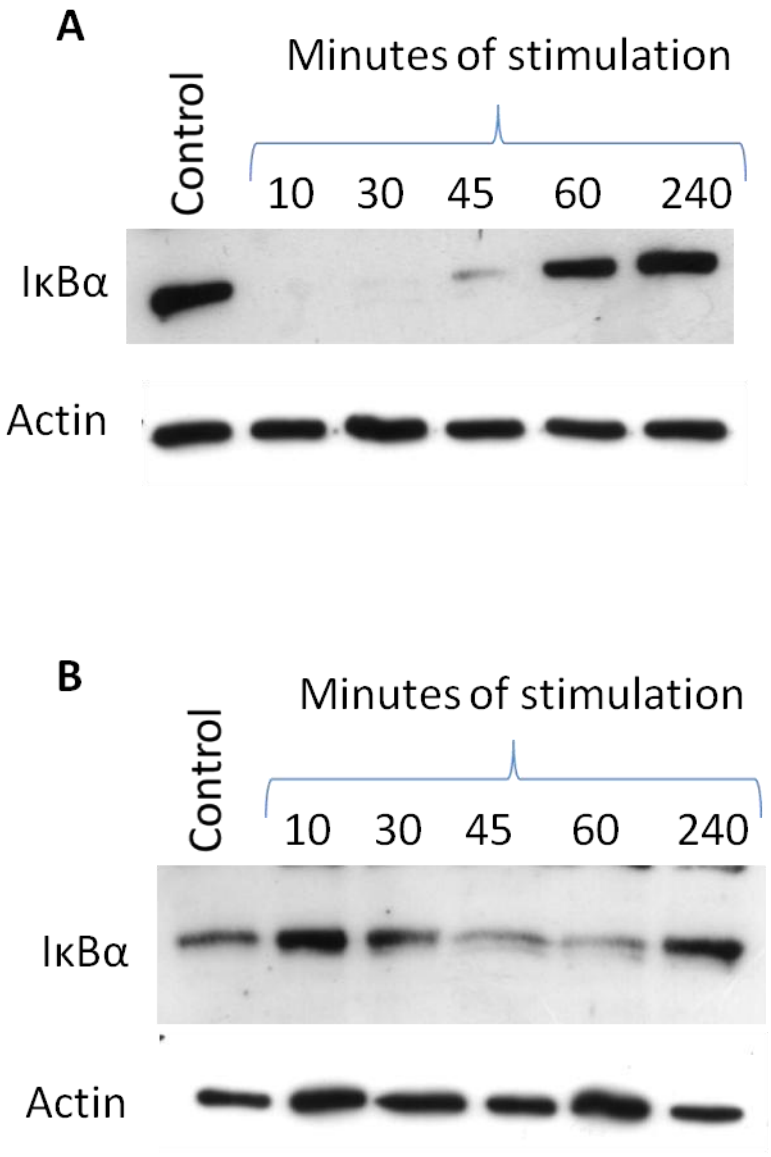


Figure 5.28: Poly(I:C) and IL-1β induced IκB-α degradation in BEAS-2B

BEAS-2Bs were stimulated with either 50 ng/ml IL-1β (**A**) or 25 µg/ml poly(I:C) (**B**) for 15-, 30-, 45-, 60- and 240-minutes and these cells were lysed and immunoblotted using antibodies to either IκBα or actin (loading control). Representative immunoblot of 2 independent experiments.

5.13 Poly(I:C) induced IκB-α degradation in primary bronchial epithelial cells

Pellino1 knockdown in PBECs shows a significant reduction in IL-8 in response to poly(I:C) and NF-κB activation is thought to be an important transcription factor related to upregulation of this chemokine. We wanted to investigate NF-κB regulation in response to poly(I:C) in PBECs.

As poly(I:C) led to IκBα degradation in BEAS-2B cells (**Figure 5.28b**), it was hypothesised that poly(I:C) would also lead to IκBα degradation in PBECs with the same kinetics. However, despite IκBα degradation occurring after 15 minutes in response to IL-1β (**Figure 5.29a**), no such degradation of IκBα could be visualised after up to 4 hours of poly(I:C) stimulation (**Figure 5.29b**).

As poly(I:C) has shown to exhibit different IκBα degradation kinetics to that of IL-1β in BEAS-2B cells, as shown in (**Figure 5.26**), it was hypothesised that the IκBα degradation event was occurring at a different timepoint to those previously used. To test this, PBECs were stimulated every 2-4 hours over a 24 hour timecourse with poly(I:C), however no IκBα protein degradation could be visualised at these timepoints (**Figure 5.30**).

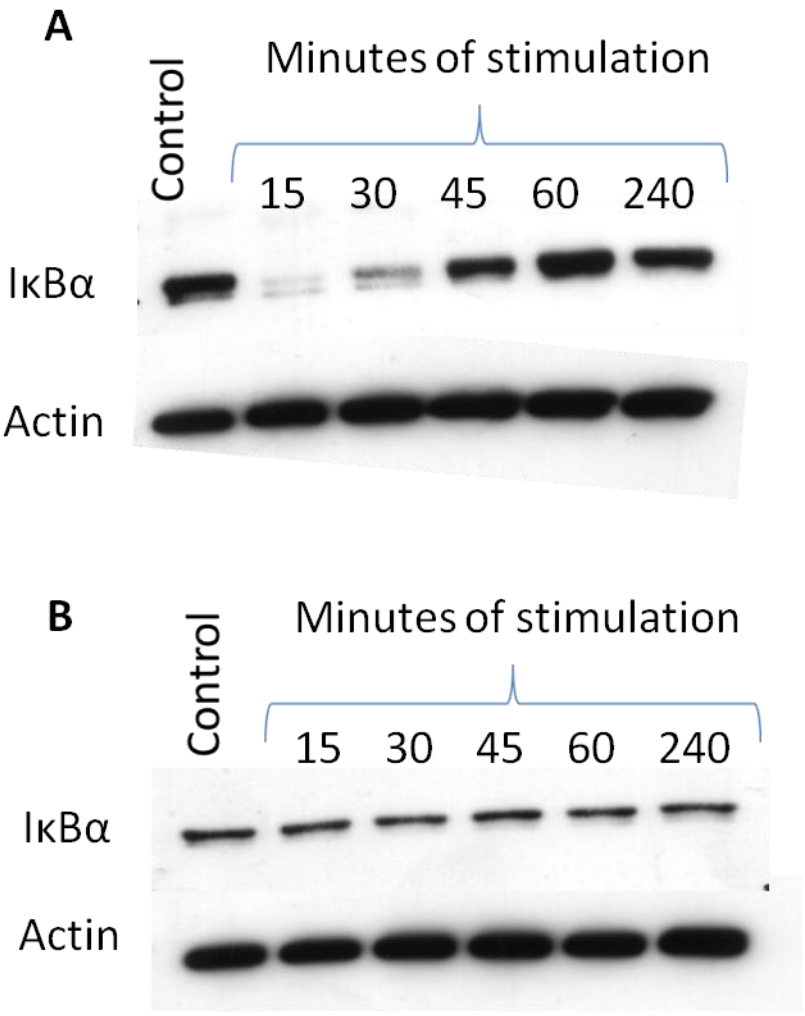


Figure 5.29: Poly(I:C) and IL-1β induced IκB-α degradation in primary bronchial epithelial cells

PBECs were stimulated with either 50 ng/ml IL-1β (**A**) or 25 µg/ml poly(I:C) (**B**) for 15-, 30-, 45-, 60- and 240-minutes. These cells were lysed and immunoblotted using antibodies to either IκBα or actin (loading control). Representative immunoblot of 2 independent experiments.

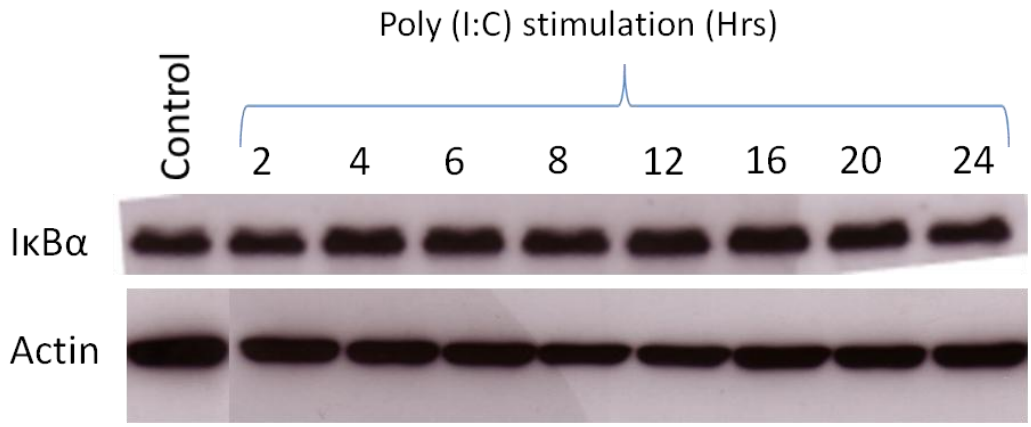


Figure 5.30: Poly(I:C) induced IκB-α degradation in PBECs over 24 hours in primary bronchial epithelial cells

PBECs were stimulated with either 25 µg/ml Poly(I:C) for 2-, 4-, 6-, 8-, 12-, 16-, 20- and 24 hours. These cells were lysed and immunoblotted using antibodies to either IκBα or actin (loading control). Representative immunoblot of 2 independent experiments.

5.11.1 Poly(I:C) induced p38 MAPK activation in primary bronchial epithelial cells

Stimulation of TLR/IL-1R signalling pathways activate a complex intracellular network of signalling molecules that leads to the activation of transcription factors such as NF-κB and AP-1. AP-1 is a dimer of c-Jun and s-Fos and acts in a synergistic manner with NF-κB p65 and leads to the activation of transcription of cytokines and growth factors (Karin 1996). AP-1 is activated by mitogen activated protein (MAP) kinases, which include the serine/threonine kinases ERK, JNK/SAPKs and p38s. TLR/IL-1R activation leads to the activation of TRAF6, which as well as leading to NF-κB activation also leads to activation of a three tier phosphorylation cascade that includes MAP kinase kinase (MAP2K) and MAP kinase kinase kinase (MAP3K) leading to activation of the ERK, JNK/SAPKs and p38s and consequentially the transcription factor AP-1. A figure summarising TLR3-mediated p38 MAPK activation can be found in **Figure 5.31**.

As poly(I:C) stimulation of PBECs did not seem to activate canonical NF-κB signalling despite high levels of IL-8 generation, it was hypothesised that poly(I:C) stimulation may lead to p38 MAPK activation to initiate the transcription of IL-8 or if p38 phosphorylation was absent in poly(I:C) stimulated cells it would support the lack of activation of canonical signalling pathways in PBECs in response to this agonist.

PBECs were stimulated with either IL-1β (control) or poly(I:C) for up to 4 hours and protein lysates were immunoblotted for active (phosphorylated) p38 (p-p38) or total p38 (loading control). Upregulation of p-p38 protein can be seen after 15 minutes of IL-1β stimulation (**Figure 5.32a**), however no upregulation of p-p38 can be seen at 15-, 30-, 45-, 60-, or 240-minutes after poly(I:C) stimulation (**Figure 5.32b**). It is note-worthy that basal phosphorylation of p38 was higher than was visualised after 1 hour of IL-1β stimulation in PBECs, suggesting that p-p38 may be actively degraded to switch off pro-inflammatory signals.

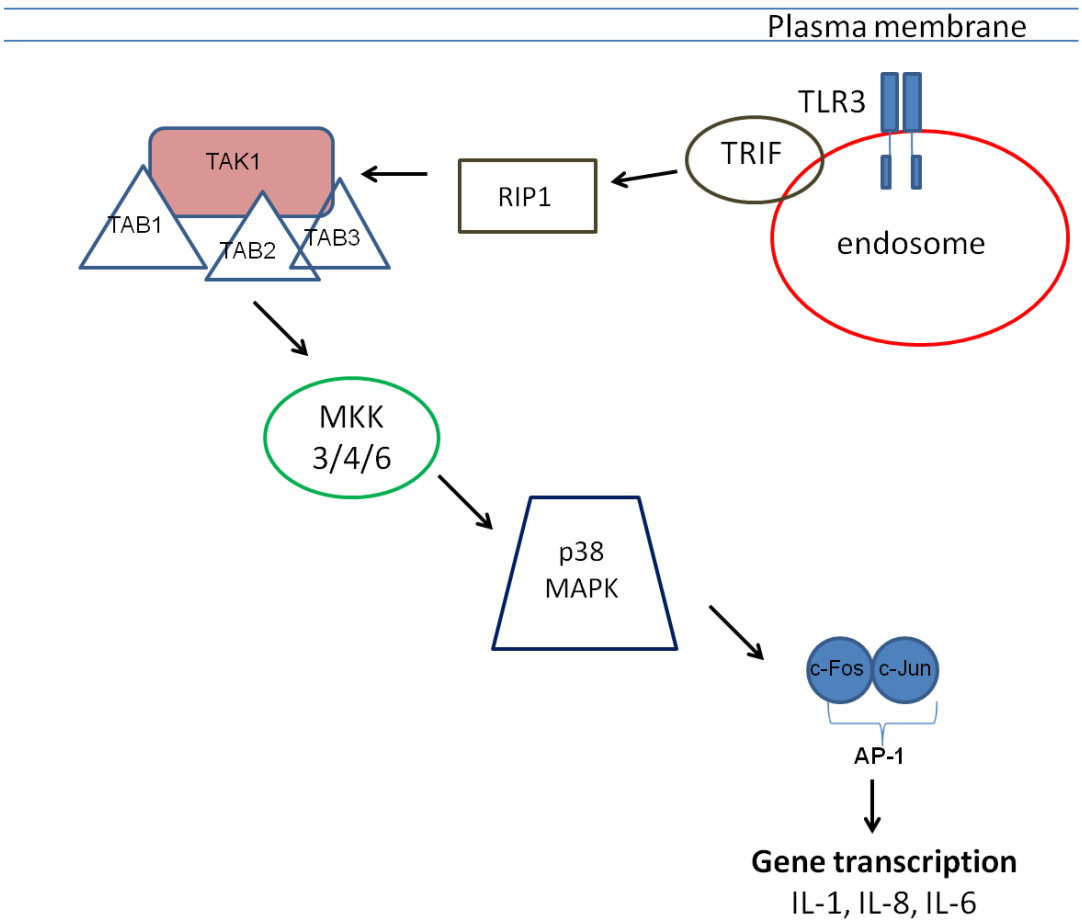


Figure 5.31: TLR3-mediated p38 MAPK activation

Activation of TLR3 by viral dsRNA in the endosome leads to the recruitment of TRIF and the subsequent activation of RIP1. RIP1 recruits and activates TAK1, which leads to the activation of the MAPK cascade; MAPKK3/4/6 are specifically involved in the activation of p38 and subsequent activation of the AP-1 transcription factor.

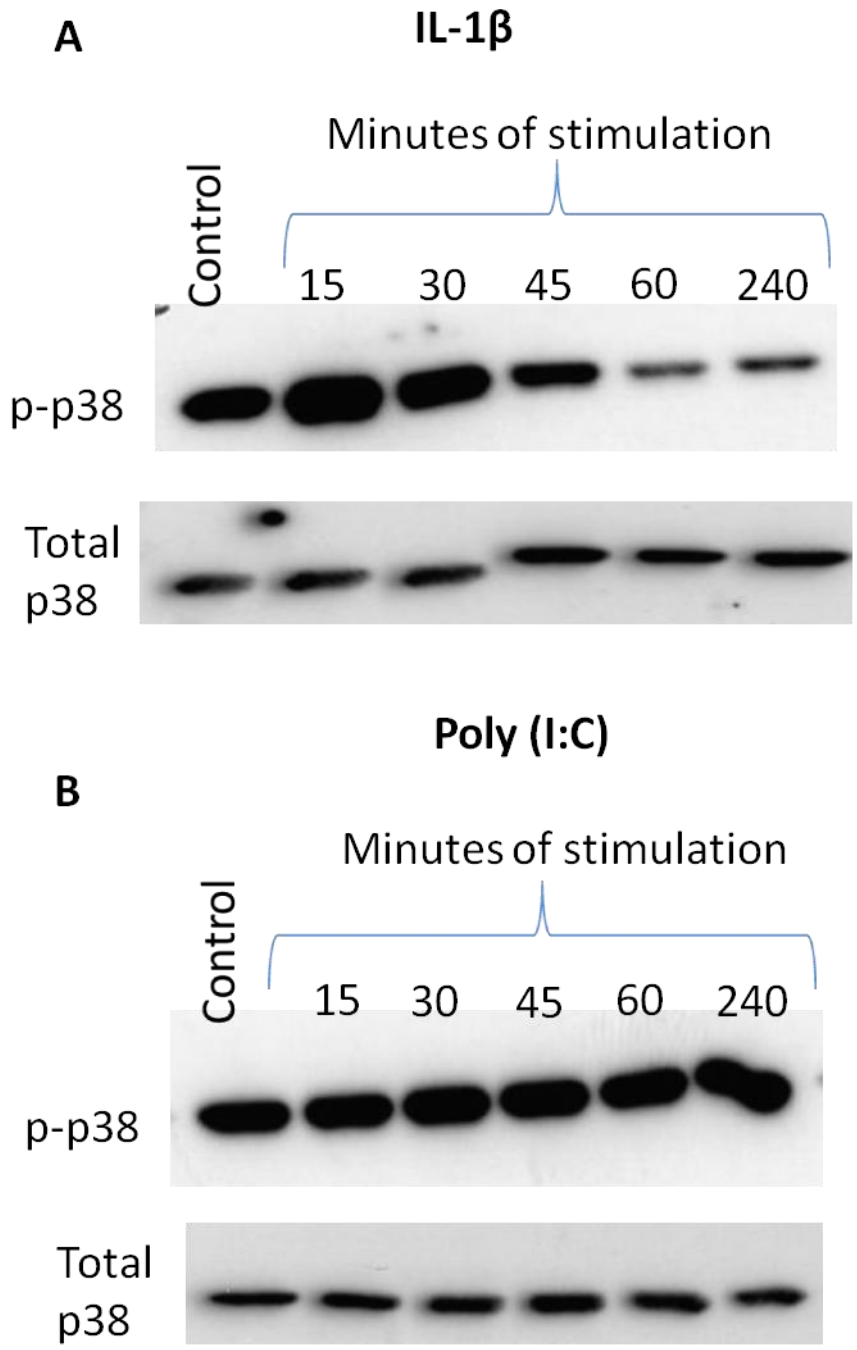


Figure 5.32: Poly(I:C) induced p38 MAPK activation in primary bronchial epithelial cells

PBECs were stimulated with either 50 ng/ml IL-1β (**A**) or 25 µg/ml poly(I:C) (**B**) for 15-, 30-, 45-, 60- and 240-minutes. These cells were lysed and immunoblotted using antibodies to either active-p38 or total-p38 (loading control). Representative immunoblot of 2 independent experiments.

5.11.2 Effect of transient Pellino1 knockdown on non-canonical NF-κB activation in primary bronchial epithelial cells

Having realised that canonical NF-κB signalling pathways did not seem to be activated in response to poly(I:C) in PBECs, we searched the literature and found limited data suggesting that non-canonical NF-κB signalling could be activated in response to viral stimuli (Rajput, Kovalenko et al. 2011). We investigated the involvement of non-canonical NF-κB activation in response to poly(I:C) stimulation in PBECs.

The term NF-κB relates to a family of 5 transcription factors, RelA (p65), RelB, c-Rel and the precursor proteins NF-κB1 (p105) and NF-κB2 (p100), all of which share the Rel homology domain that is responsible for dimerisation and DNA binding. RelA (p65), RelB and c-Rel are sequestered in the cytoplasm by the inhibitory IκB proteins in the canonical NF-κB signalling pathway, however in the non-canonical NF-κB signalling pathways, p100 and p105 are unable to translocate to the nucleus until activation of the pathway leads to partial or complete proteolytic cleavage of the precursor protein which releases the active p50 and p52 proteins, respectively (Rice, MacKichan et al. 1992; Mercurio, DiDonato et al. 1993; Pomerantz and Baltimore 2002; Solan, Miyoshi et al. 2002).

Poly(I:C) stimulation of PBECs did not seem to activate canonical NF-κB (**Figure 5.29 + 5.30**) or p38 MAPK signalling pathways (**Figure 5.32**), so the ability of poly(I:C) to activate the non-canonical NF-κB signalling pathway was explored using western blotting. The transcription factor RelB is held in the inactive form in the cytoplasm by the precursor protein p100; upon activation p100 is partially degraded, thus releasing the active dimer of RelB and p52 (Fong and Sun 2002; Amir, Haecker et al. 2004; Cohen, Achbert-Weiner et al. 2004). The antibody used detected both inactive (p100) and active (p52) forms of NF-κB2 and pilot data suggest that after 16-24 hours of poly(I:C) stimulation there was an increase in both inactive and active forms of NF-κB2 (**Figure 5.34**). A possible signalling pathway connecting the TLR3 and the non-canonical NF-κB pathways can be found in **Figure 5.33**.

We hypothesised that the reduced IL-8 levels seen in Pellino1 knockdown PBECs following poly(I:C) treatment was a result of decreased non-canonical NF-κB activation. The effect of Pellino1 knockdown on non-canonical NF-κB activation was explored using western blotting. Pilot data suggests that Pellino1 knockdown PBECs stimulated with poly(I:C) for 24 hours show a decreased activation of non-canonical NF-κB activation compared to the scrambled siRNA transfected counterpart (**Figure 5.35**).

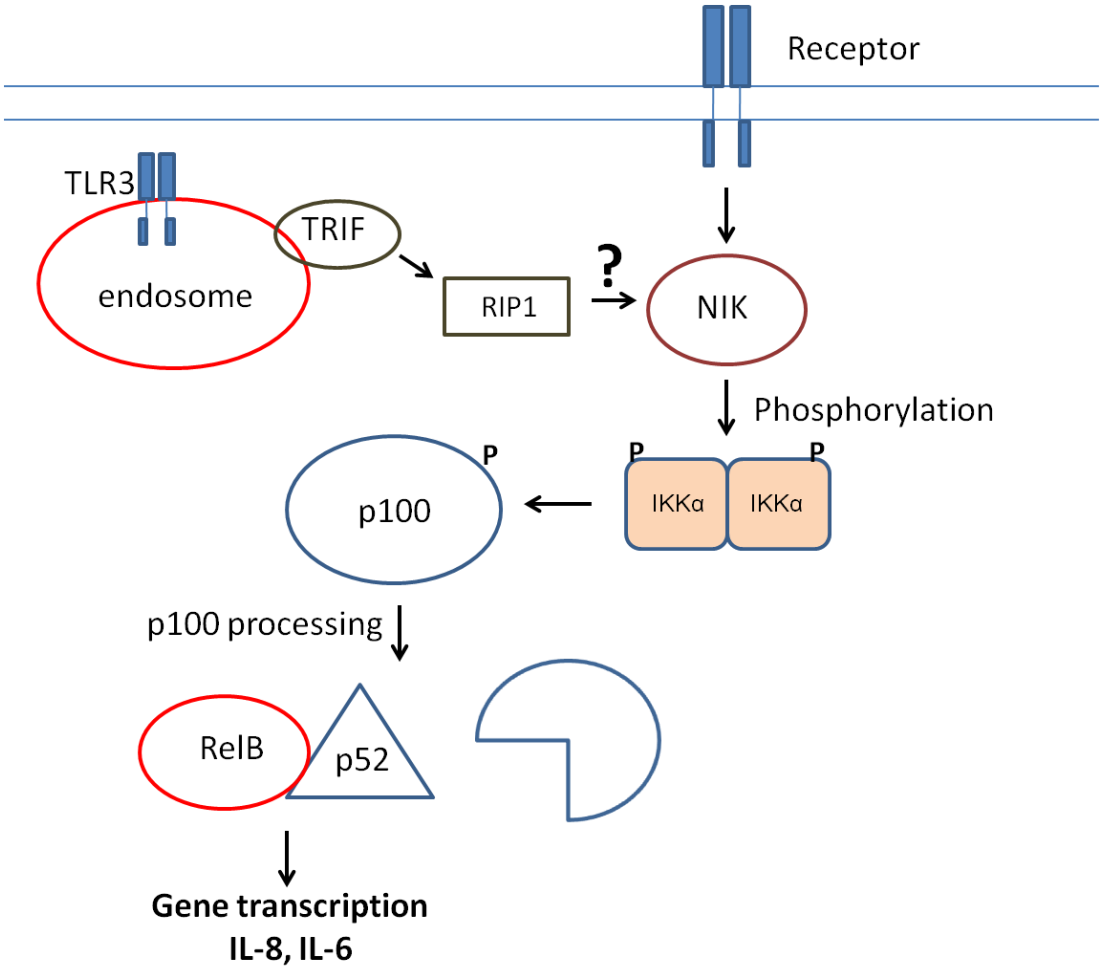


Figure 5.33: A possible signalling pathway connecting TLR3 to non-canonical NF-κB signalling

A possible signalling pathway that connects the TLR3 pathway to non-canonical NF-κB activation is described in the figure. Activation of TLR3 by viral dsRNA in the endosome leads to the recruitment of TRIF and the subsequent activation of RIP1. Activated RIP1, or perhaps as yet uncharacterised mediators, could activate NIK, which leads to activation of IKKα homodimers and subsequent p100 processing. Active p52 fragments can dimerise with RelB and translocate to the nucleus where they can upregulate target genes including IL-8 and IL-6.

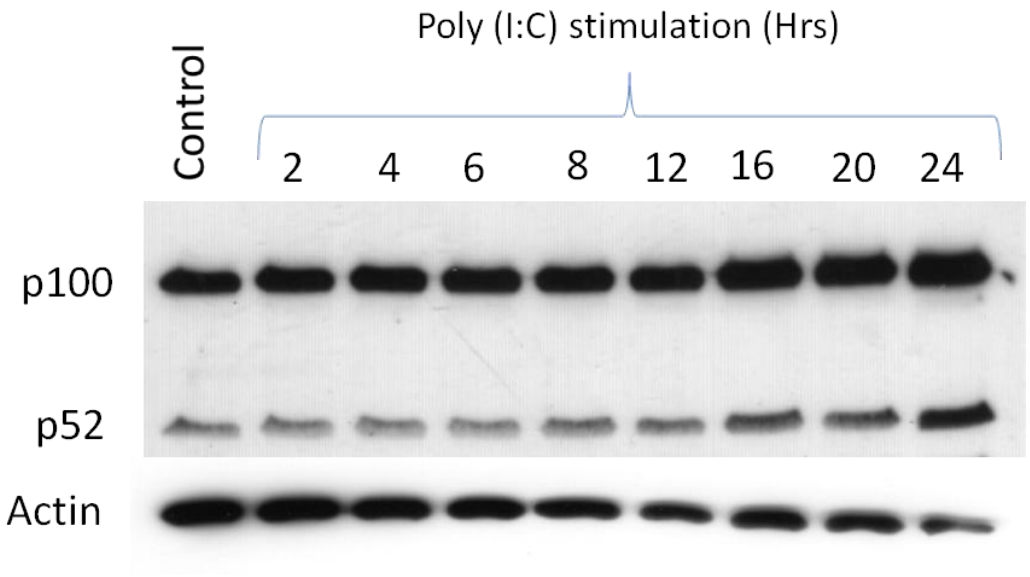


Figure 5.34: Poly(I:C) induced non-canonical NF-κB activation in primary bronchial epithelial cells over 24 hours

PBECs were stimulated with 25 µg/ml Poly(I:C) for 2-, 4-, 6-, 8-, 12-, 16-, 20- and 24 hours. These cells were lysed and immunoblotted using antibodies to either p100/p52 or actin (loading control). Representative immunoblot of 2 independent experiments.

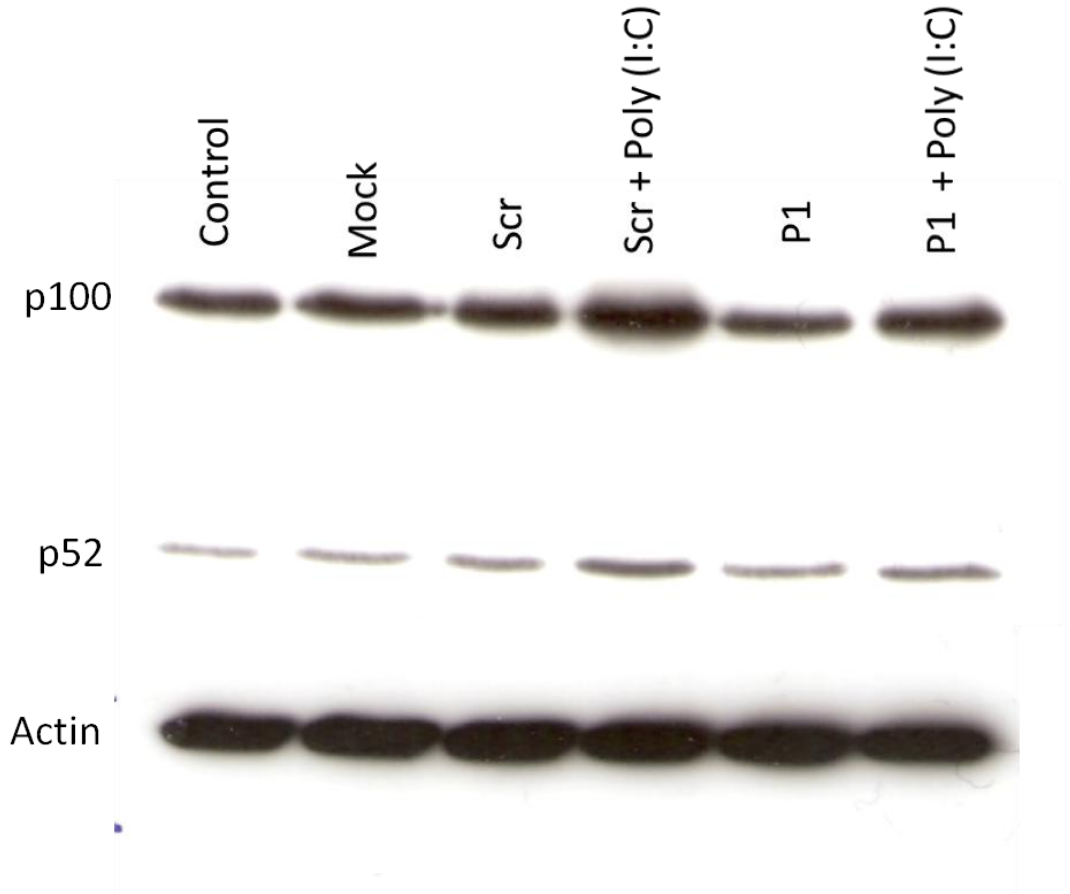


Figure 5.35: Effect of transient Pellino1 knockdown on non-canonical NF-κB activation in primary bronchial epithelial cells over 24 hours

PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and these cells were stimulated with 25 μg/ml poly(I:C) for 24 hours. These cells were lysed and immunoblotted using antibodies to either p100/p52 or actin (loading control). Representative immunoblot of 2 independent experiments.

5.12 Effect of Pellino1 knockdown on induced cytokine release in Asthmatic patient primary bronchial epithelial cells

Asthma is one of the worlds' most prevalent respiratory diseases and affects over 5 million people in the UK. Respiratory viruses, such as rhinovirus, are a major cause of asthma exacerbations (Nicholson, Kent et al. 1993; Johnston, Pattemore et al. 1995). Previous data has shown that Pellino1 knockdown in PBECs leads to a reduction in IL-8 and IL-6 and preserved RANTES and IP-10 release in response to both the viral mimic poly(I:C) (**Figure 5.9 - 5.11**) and the natural pathogen RV1B (**Figure 5.21 – 5.24**). In collaboration with Irene H Heijink's laboratory at the University Medical Center in Groningen, PBECs isolated from asthmatic patients were used to measure the effect of Pellino1 knockdown on IL-1β, poly(I:C) and TNFα induced cytokine release.

Pellino1 was knocked down in asthmatic patient PBECs using targeted siRNA (**Figure 5.36**) and Pellino1 knockdown led to reduced IL-8 production in response to poly(I:C) **Figure 5.37b**). IL-1β and TNFα responses were unaffected by Pellino1 knockdown (**Figure 5.37a+c**). It is also important to note that asthmatic patient samples had approximately 10 times higher baseline levels of IL-8 release compared to normal PBECs. RANTES production in asthmatic patient PBECs was unaffected by Pellino1 knockdown (**Figure 5.38**). The siRNA experiments were carried out in Irene H Heijink's laboratory, University Medical Center Groningen and the ELISAs and qPCR was carried out in our own laboratory.

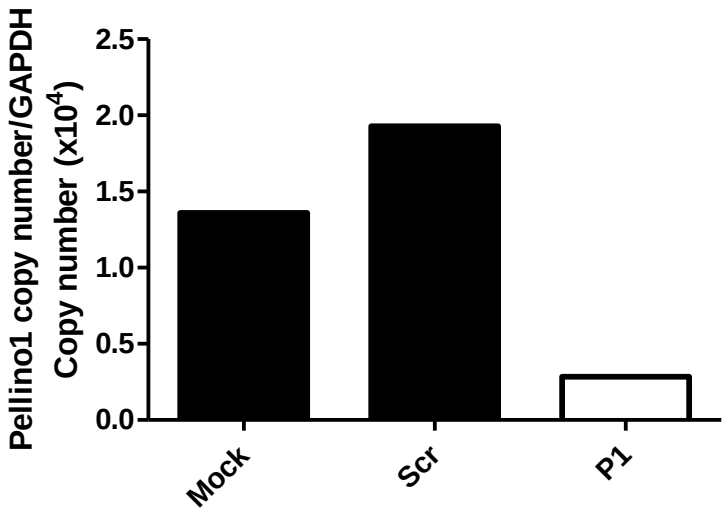


Figure 5.36: Pellino1 mRNA knockdown in asthmatic patient primary bronchial epithelial cells

Asthmatic patient PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA. Total RNA was extracted from these cells 48 hours post-transfection and converted into cDNA. Quantitative PCR was used to compare Pellino1 mRNA expression levels in mock and scrambled siRNA (Scr) transfected to that of Pellino1 knockdown PBECs. Pellino1 copy number was normalised to GAPDH copy number as a loading control and data are expressed as a ratio of Pellino1 copy number normalised to GAPDH (control) copy number from a single experiment. (Lysates were kindly provided by Irene H Heijink, University Medical Center Groningen.)

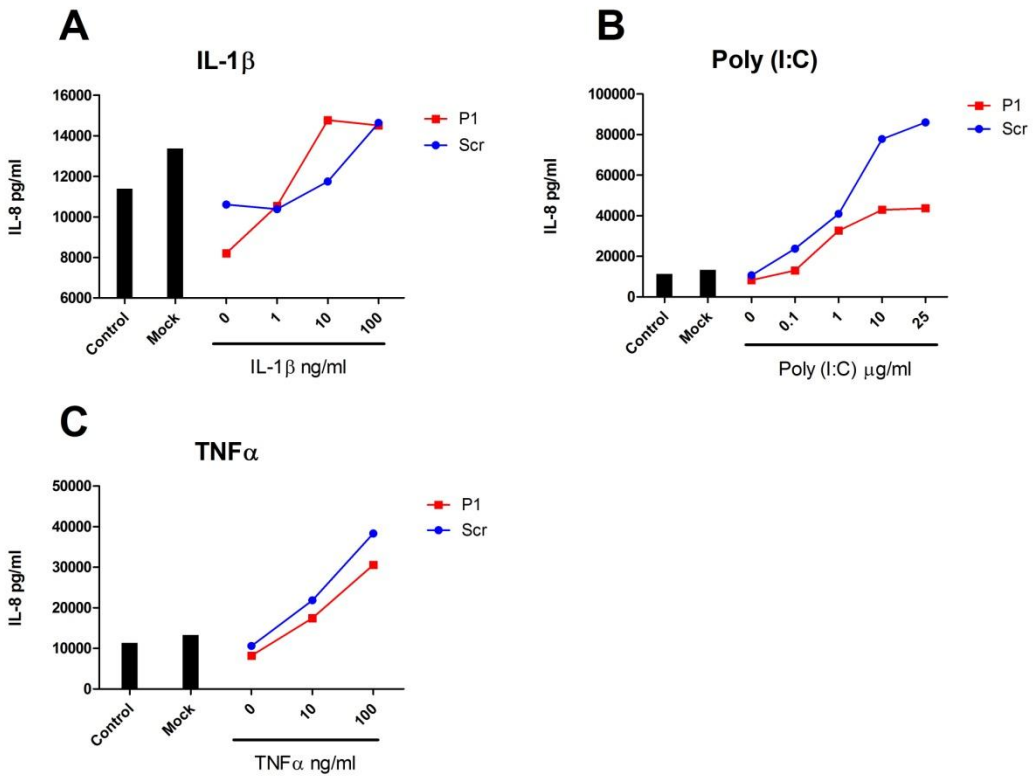


Figure 5.37: IL-8 generation in Pellino1 knockdown asthmatic patient primary bronchial epithelial cells in response to IL-1β, poly(I:C) and TNFα

Asthmatic patient PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of IL-1β (**A**), TNFα (**B**), poly(I:C) (**C**) or media (**A-C**). Untransfected (Utx) and mock transfected asthmatic patient PBECs cells were treated with media only. Supernatants were collected and analysed for IL-8 generation by ELISA. Data are expressed as pg/ml IL-8 from a single experiment. (Supernatants were kindly provided by Irene H Heijink, University Medical Center Groningen.)

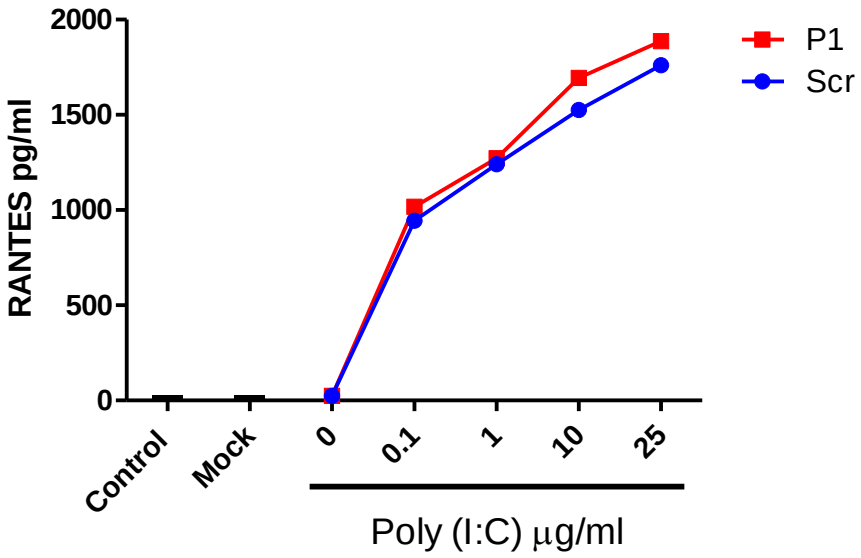


Figure 5.38: RANTES generation in Pellino1 knockdown asthmatic patient primary bronchial epithelial cells in response to poly(I:C)

Asthmatic patient PBECs were transiently transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and were treated for 24 hours with a concentration response of poly(I:C) or media. Untransfected (Utx) and mock transfected asthmatic patient PBECs cells were treated with media only. Supernatants were collected and analysed for RANTES generation by ELISA. Data are expressed as pg/ml RANTES from a single experiment. Supernatants were kindly provided by Irene H Heijink, University Medical Center Groningen.

5.14 Summary

Transient transfection of Pellino1-targeted siRNA into HeLa, BEAS-2B cells and PBECs leads to the marked reduction of Pellino1 at the mRNA level. Pellino1 knockdown HeLa cells show a reduction in both IL-8 and RANTES in response to poly(I:C), however Pellino1 knockdown had no effect on IL-8 production in response to IL-1β. Pellino1 knockdown in BEAS-2B cells led to the significant reduction of IL-8 in response to both IL-1β and Poly(I:C), however RANTES was unaffected by Pellino1 knockdown and IFN production was only slightly reduced in Pellino1 knockdown BEAS-2B cells.

Similarly to HeLa and BEAS-2B cells, transient knockdown of Pellino1 in PBECs led to the significant reduction of IL-8 in response to poly(I:C), however in contrast to BEAS-2B cells IL-1β signalling was preserved in Pellino1 knockdown PBECs. Other NF-κB-regulated cytokines were also reduced in response to poly(I:C) including IL-6, TNFα, GM-CSF, MCP-1, MIP-1α and MIP-1β. Although Pellino1 knockdown in PBECs had no effect on Poly(I:C)-induced RANTES production, another interferon-stimulated gene IP-10 was significantly reduced but only at the highest dose of poly(I:C) and IFN production was slightly reduced in poly(I:C)-stimulated Pellino1 knockdown PBECs.

RIP1 has been suggested as a putative target for the E3 ubiquitin ligase activity of Pellino1 (Chang, Jin et al. 2009) and it was therefore hypothesised that transient RIP1 knockdown in PBECs would recreate the Pellino1 knockdown phenotype. Interestingly however, RIP1 knockdown PBECs, in contrast to Pellino1 knockdown, exhibited significantly increased levels of IL-8 generation in response to poly(I:C).

As IL-8 production was profoundly reduced in response to the viral mimic poly(I:C) in Pellino1 knockdown PBECs, cytokine production was then measured in Pellino1 knockdown PBECs that had been infected with the natural viral pathogen RV1B. As hypothesised, Pellino1 knockdown PBECs show a significant decrease in NF-κB-regulated cytokines IL-8 and IL-6 whereas both the interferon-stimulated genes RANTES and IP-10 and IFN production were unaffected by Pellino1 knockdown. There was also no significant difference in viral copy number between Pellino1 knockdown and scrambled control transfected PBECs.

Viral signalling pathways were investigated in BEAS-2B cells and PBECs using western blot. It was found that BEAS-2B cells activate canonical NF-κB signalling in response to poly(I:C) as IκBα degradation could be visualised after 45 minutes of poly(I:C) stimulation, however

this degradation event could not be visualised in PBECs at the same timepoints or over a 24 hour timecourse. This lack of IκBα degradation in PBECs led to the suggestion that these cells activate a different signalling pathway to canonical NF-κB signalling in response to TLR3 activation and thus activation of the non-canonical NF-κB signalling pathway in response to Poly(I:C) was investigated in PBECs. The non-canonical (NF-κB2) signalling pathway is activated by p100 processing to an active p52 fragment that dimerises with RelB and translocates to the nucleus to initiate target gene transcription. Pilot data suggested that both the active (p52) and inactive (p100) forms of NF-κB2 were upregulated after 16 hours of poly(I:C) treatment of PBECs and preliminary data suggest that this upregulation is perturbed in Pellino1 knockdown PBECs stimulated with poly(I:C).

Pellino1 may prove to be a useful drug target for the treatment of inflammatory diseases including asthma where infections with rhinovirus have shown to be a major cause of exacerbations of this disease. Data presented in this thesis suggest that Pellino1 could be targeted to downregulate neutrophilic inflammation in response to rhinovirus whilst retaining antiviral immunity to overcome the initial infection. To test whether Pellino1 is a key mediator in diseased lung airway epithelium, Pellino1 knockdown PBECs from asthmatic patients were stimulated with IL-1β, poly(I:C) and TNFα and preliminary results suggest that IL-8 is reduced in Poly(I:C) stimulated asthmatic PBECs, however IL-1β and TNFα responses and RANTES production are unaffected. It is also important to note however, the baseline of IL-8 production was approximately 10x higher than is measured in normal PBECs.

Chapter 6 – Discussion

Inflammation is an important host response that is necessary for the efficient clearance of pathogens and repair of sterile tissue damage, however dysregulation of inflammation is associated with various inflammatory diseases including asthma and COPD. The inflammatory response is regulated by a great number of mediators, a family of which include the Pellino family. Pellinos are a family of E3 ubiquitin ligases that have been shown to be involved in the ubiquitination of the key mediators of the TLR/IL-1R signalling pathway. The functional significance of Pellinos in inflammation in human lung airway epithelium is still yet to be elucidated. We think that these are important cells to study as they are often the first cell-type to be exposed to inhaled pathogens and have been shown to play a dynamic role in the immune response.

We have been particularly interested in Pellino1, as at the start of this thesis it was thought to be involved in regulating the IL-1 signalling pathway (Jiang, Johnson et al. 2003) and more recently has been shown to be important in viral signalling (Chang, Jin et al. 2009). The latter was of particular interest as rhinovirus infection is a major cause of asthma exacerbations (Nicholson, Kent et al. 1993; Johnston, Pattemore et al. 1996) and therefore Pellino1 could be a potential drug target. This thesis investigated the expression and regulation of the Pellino family in human cells and the functional importance of Pellino1 in inflammation.

6.1 Expression and regulation of Pellinos

6.1.1 Differential expression of Pellinos in human tissue cells and primary leukocytes

The Pellino family is differentially expressed in human tissue cells and primary human leukocytes. Messenger RNA from all of the Pellino family could be detected after 30 PCR amplification cycles in the human tissue cell-lines HeLa and HEK and in primary peripheral blood monocytes. However, in the lung airway epithelial cell-line BEAS-2B and primary bronchial epithelial cells, no Pellino2 transcript could be detected. Primary peripheral blood neutrophils, express mRNA for all Pellinos except Pellino3a which is barely detectable after 30 PCR amplification cycles.

Previous data support the finding of tissue-specific Pellino expression as it has been shown that although Pellinos are ubiquitously expressed, the relative levels of expression of individual Pellinos in different organs significantly differ. Expression of Pellino1 and Pellino2

has previously been described in the mouse, where Pellino1 was found to be highly expressed in peripheral blood leukocytes; moderately expressed in placenta, lung, liver, kidney, spleen, thymus, skeletal muscle and brain; and only expressed at low levels in small intestine, colon and heart (Jiang, Johnson et al. 2003). Pellino2 transcripts could be detected at high levels in murine tissues associated with the innate immune response (skin and liver) and testis, however were either undetectable or expressed at very low levels in other tissues (Yu, Kwon et al. 2002). Human Pellino3 transcripts are found at high levels in brain, heart and testis; in lower levels in kidney, liver, lung, placenta, small intestine, spleen and stomach; and barely detectable in colon and muscle tissue (Jensen and Whitehead 2003). However human Pellino3 expression patterns did not distinguish between the splice variants Pellino3a and Pellino3b.

Each Pellino has been shown to function in a non-redundant manner and they play defined roles in the TLR/IL-1R signalling pathway. Pellino1 is thought to play a key role in NF-κB activation, either through binding of the IRAKs (Jiang, Johnson et al. 2003) or more recently with the generation of the Pellino1 knockout mouse, it has been suggested that it is involved in TLR3 signalling (Chang, Jin et al. 2009). Pellino1 deficient mice develop normally but are resistant to LPS-induced toxic shock and show impaired B-cell proliferation and induction of co-stimulatory molecules CD86 and MHC class II molecules. In contrast to previous studies, Pellino1 deficient MEFs showed preserved IL-1 signalling, however had impaired responses to the TLR3 agonist and viral mimic poly(I:C) (Chang, Jin et al. 2009). This study also showed that Pellino1 was capable of binding to and ubiquitinating RIP1 and this was suggested as a potential Pellino1 target in TLR3 signalling (Chang, Jin et al. 2009). Pellino1 has also been linked to the transcription factor IRF3, where TBK and IKKε phosphorylate Pellino1 leading to the activation of its E3 ligase activity and this was dependent on the presence of IRF3 (Smith, Liu et al. 2011).

Pellino3b has been shown to act as a negative regulator of IL-1-induced NF-κB activation (Xiao, Qian et al. 2008). It is interesting to note that Pellino1 and Pellino3b that are involved in the fundamental processes of NF-κB regulation are expressed in all human cell types that were tested. Pellino2 and Pellino3a, which have both been implicated in MAPK activation (Jensen and Whitehead 2003; Butler, Hanly et al. 2005), show differential expression; Pellino2 was not expressed in lung airway epithelial cells and Pellino3a was not expressed in primary human neutrophils.

It is likely that the repertoire of Pellino proteins that each cell-type expresses reflects its function and therefore its requirement for regulatory mediators of inflammation. The monocyte, which is involved in responding to pathogen and synthesising pro-inflammatory cytokines, express all of the Pellino family, whereas non-haematopoietic lung epithelial cells and non-synthetic neutrophils express only 1 of either Pellino2 or Pellino3a. Cell-lines that have lost much of their original cellular identity through transformation into immortalised cells and extensive culture time, such as HeLa and HEK cells, may have lost the expression pattern of the Pellino family that would be expressed in human primary cervical or embryonic kidney cells and as such, express the entire Pellino family.

It is important to note that the immortalised cell-line BEAS-2B, which retains many of the cellular markers of lung airway epithelial cells and retain the ability to undergo spontaneous squamous differentiation in response to serum, and primary bronchial epithelial cells have the same expression pattern of Pellinos. This indicates that in terms of Pellino experiments, BEAS-2B may be a good representative cell-line in which to study the roles of Pellinos in airway epithelial cells.

6.1.2 Regulation of Pellino family in response to TLR/IL-1R agonists

Expression of the Pellino family is tissue specific, however this expression pattern may be altered in response to a pro-inflammatory stimulus. We, and others (Smith, Liu et al. 2011), have found that commercially available antibodies do not detect members of the Pellino family. We tested the two commercially available antibodies to Pellino1 (Abcam and Santa Cruz) and found that these antibodies did not show protein knockdown when the mRNA levels were significantly reduced (data not shown) and each antibody bound to different compartments when cells were fractionated into cytosolic and membrane extracts. Considering these data, Pellino regulation was analysed at the transcriptional level.

The limitation of using RT-PCR to measure regulation of Pellinos in response to pro-inflammatory stimuli is that it is not robustly quantitative and therefore is difficult to detect small changes in mRNA levels. However, induction of expression and large-scale differences in mRNA expression can be visualised using RT-PCR. No induction of Pellino2 or Pellino3a in response to IL-1β, LPS or TNFα for 1 or 3 hours can be visualised in lung airway epithelial cells and neutrophils, respectively. However Pellino3b mRNA levels appear to decrease in BEAS-2B cells upon stimulation with pro-inflammatory agonists, which supports evidence that it acts as a negative regulator of NF-κB (Xiao, Qian et al. 2008) and thus may be down-regulated during inflammation. This finding is supported by previous work by Butler *et al* in

2007 who found that Pellino3 protein was degraded in response to LPS treatment in human PBMCs (Butler, Hanly et al. 2007). However, downregulation of Pellino3b mRNA is not seen in PBECs, monocytes or neutrophils in response to IL-1β, LPS or TNFα.

In contrast to RT-PCR, qPCR is a highly quantitative methodology and was used to analyse Pellino1 expression in key experiments. Quantitative PCR was used to measure the level of Pellino1 mRNA in response to proinflammatory stimuli in neutrophils, BEAS-2B and PBECs. Preliminary data suggested that Pellino1 mRNA was upregulated after 3 hours of IL-1β, LPS and TNFα treatment in neutrophils. Upregulation of Pellino1 was seen in PBECs after 1 and 3 hours of IL-1β and TNFα or 3 hours of poly(I:C) treatment, however LPS had no effect on Pellino1 levels in PBECs at these timepoints. These preliminary data are supported by previous studies that show that Pellino1 mRNA is upregulated in bone-marrow-derived macrophages (BMDMs) after 3 hours of Lipid A (component of LPS) stimulation (Weighardt, Jusek et al. 2004) and more recently, a group that made a Pellino1-specific antibody showed that Pellino1 protein is greatly enhanced in BMDMs that are stimulated with LPS or poly(I:C) for 3-6 hours (Smith, Liu et al. 2011). The upregulation of Pellino1 in response to pro-inflammatory stimuli may indicate roles in NF-κB signalling.

Interestingly, in contrast to neutrophils and PBECs, BEAS-2B cells showed a slight reduction of Pellino1 in response to IL-1β, poly(I:C) and TNFα stimulation and RV1B infection. This finding may reflect the differences between primary and immortalised cell phenotypes. However, PBECs also did not show an upregulation of Pellino1 in response to LPS and therefore this may suggests that Pellino1 regulation is more complex than it merely being upregulated in response to inflammation by activation of NF-κB or other transcription factors and other mediators may be required for its transcriptional activation.

Pellino1 may also be regulated at the translational level or by post-translational modifications such as phosphorylation (Smith, Peggie et al. 2009), ubiquitination (Smith, Peggie et al. 2009), or sumoylation (Kim, Sung et al. 2011). Linkage-specific ubiquitin antibodies will be used to measure the type of ubiquitination of Pellino1 and its targets.

6.1.3 Implications of Pellino1 regulation

Preliminary data suggests that Pellino1 is upregulated at the mRNA level in response to selected pro-inflammatory stimuli in primary human cells. As Pellino1 is upregulated in response to IL-1β stimulation in PBECs, this may support previous work that has shown that it is involved in the ubiquitination of IRAK and subsequent NF-κB activation in human cells (Jiang, Johnson et al. 2003). However other cell-types tested, BEAS-2B cells, primary human

neutrophils and monocytes, did not show an increase in Pellino1 in response to IL-1β. These data could suggest that Pellino1 is dispensable for IL-1 signalling, which has been previously described in the Pellino1$^{-/-}$ mouse (Chang, Jin et al. 2009), or that constitutive Pellino1 levels are sufficient to activate signalling in these cell types.

Pellino1 is upregulated in response to the TLR3 agonist poly(I:C) after 3 hours of stimulation in PBECs , which may reflect data from the Pellino1$^{-/-}$ mouse that has shown that Pellino1 is involved in TRIF-dependent signalling (Chang, Jin et al. 2009) and is therefore upregulated in response to activation of the pathway it regulates. A recent study has that has created a Pellino1-specific antibody have also shown that Pellino1 protein is upregulated in response to poly(I:C) stimulation in murine bone marrow-derived macrophages (BMDMs) (Smith, Liu et al. 2011).

In contrast to PBECs, poly(I:C) stimulation or RV1B infection leads to the slight reduction of Pellino1 in BEAS-2B cells, which could suggest that Pellino1 could function as a negative regulator of TLR3 signalling in these cells and therefore is down-regulated to remove suppression of TLR signalling in the presence of pro-inflammatory stimuli. Pellino1 has previously been described as a negative regulator in T-cell activation (Chang, Jin et al. 2011).

Data from the Pellino1$^{-/-}$ mouse that has shown that Pellino1 is involved in TRIF-dependent signalling (Chang, Jin et al. 2009), which along with its involvement in the TLR3 pathway, TRIF is also involved in TLR4 signalling in response to LPS stimulation. Pellino1 protein has been shown to be upregulated in response to LPS in murine BMDMs (Smith, Liu et al. 2011) and seems to be upregulated at the mRNA level in primary human neutrophils after 3 hours of LPS. However this upregulation is not seen with LPS-stimulated PBECs. These data may reflect the differences in downstream signalling events to LPS stimulation in different cells types, as data from this thesis has shown that PBECs do not activate canonical NF-κB proteins in response to poly(I:C) stimulation.

Stimulation with the pro-inflammatory stimuli IL-1β, LPS and TNFα seemed to lead to a decrease in Pellino3b transcripts in BEAS-2B cells and this finding would support previous data that has found that Pellino3b acts a negative regulator of IL-1 signalling (Xiao, Qian et al. 2008). However, RT-PCR data is difficult to quantify and this finding was not found in any of the primary human cells tested.

It is important to note that data exploring the expression and regulation of Pellinos is still in preliminary stages and further experiments are required to establish the physiological occurrence and relevance of Pellino regulation, which could be achieved using antibodies to measure not only Pellino levels in response to stimulation, but also its phosphorylation, sumoylation and ubiquitination status. Preliminary data presented in this thesis suggest that Pellino regulation, like Pellino expression, is both cell-type and stimulus specific.

6.2 BEAS-2B stable MyD88 knockdown – clone 8

MyD88 has been shown to be a crucial adaptor in TLR-IL-1R signalling via TIR domain interactions that result in downstream signalling. All TLRs and IL-1R, except TLR3, signal through MyD88. In MyD88 deficient mice, responses to IL-1, TLR2 or TLR9 agonists were severely impaired (Adachi, Kawai et al. 1998; Kawai, Adachi et al. 1999; O'Neill, Dunne et al. 2003), however these mice were able to respond to LPS in a late-phase response (Kawai, Adachi et al. 1999), which is due to TLR4 being able to signal through both MyD88 and TRIF (Yamamoto, Sato et al. 2003).

The role of MyD88 in lung airway epithelial cells was explored by creating a stable MyD88 knockdown BEAS-2B cell-line. The stable line was created using a lentivirus carrying shRNA targeting MyD88 and cells that had incorporated the plasmid encoding the shRNA were selected (on the basis of Zeocin^{TM} resistance) and single-cell cloned. Several clones (clones 1, 7, 8, 10, 12 and 16) exhibited knockdown at the mRNA level measured by RT-PCR and two of these clones (clones 8 and 10) were verified using qPCR. The clone that was more efficient in causing MyD88 knockdown, BEAS-2B MyD88 clone 8, was used to test the functional significance of MyD88 in TLR/IL-1R signalling.

BEAS-2B MyD88 clone 8 retained its MyD88 mRNA knockdown phenotype throughout the functional experiment, suggesting that the plasmid encoding the shRNA is not silenced and transcription of the shRNA leads to MyD88 knockdown in a stable manner. As expected, stimulation of MyD88 stable knockdown BEAS-2B cells with IL-1β led to the significant reduction of IL-8 generation compared to wild-type BEAS-2Bs, whereas stimulation of these cells with the TLR3 agonist, poly(I:C), or an agonist that signals through an unrelated pathway, TNFα, show preserved IL-8 production. These data are in keeping with previous data suggesting MyD88 is essential in recruiting IRAK-4 to the receptor complex and such

activating downstream signalling leading to NF-κB activation and thus IL-8 production (Muzio, Ni et al. 1997; Li, Cousart et al. 2000; Li, Strelow et al. 2002).

TLR3 has previously been shown not to signal through MyD88 and this is supported by the finding that stimulation of MyD88 knockdown BEAS-2B cells with the TLR3 agonist poly(I:C) showed preserved IL-8 production. However, a previous study has found that MyD88 may act as a negative regulator of TLR3 signalling as IL-6 induction in response to poly(I:C) was increased in mouse MyD88$^{-/-}$ macrophages (Kenny, Talbot et al. 2009). This negative regulatory role was not seen in stable MyD88 knockdown BEAS-2Bs and this may be due to the differences in cell function as the crucial immune leukocyte macrophages, unlike airway epithelium, are highly responsive to IL-1 and other pro-inflammatory stimuli and thus require negative regulation of TLR/IL-1R signalling pathways to restrict unnecessary damage to the host. This hypothesis is supported by another study carried out in MyD88$^{-/-}$ mice showing that IL-6 and TNFα production in the lung is unchanged compared to wild-type mice after intra-tracheal injection of poly(I:C) (Chun, Liles et al. 2010).

Due to time constraints and the continued interest in Pellino1, further characterisation of the MyD88 knockdown BEAS-2B cell-line was carried out by a colleague in our group. It was found that infection of MyD88 knockdown BEAS-2B cells with the human viral pathogen RV1B led to a significant reduction in IL-8 generation, which may be due to a reduction IL-1 acting in autocrine (Stokes, Ismail et al. 2011).

Knockdown of MyD88 exhibited the expected phenotype in BEAS-2B cells and supports previous data describing the role of MyD88 in TLR/IL-1R signalling. MyD88 has been shown to be crucial for IL-1 signalling, however is dispensable in TLR3 and TNFα signalling, both of which do not utilise MyD88 for downstream signalling. These data suggested that as a proof of principle, the transduction of shRNA using lentiviral vectors and subsequent single-cell cloning was an effective method for the generation of stable target gene knockdowns in BEAS-2B cells and therefore this method was used to create Pellino1 stable knockdown clones.

6.3 HeLa stable Pellino1 knockdown – clone HL-C12

The function of Pellino1 in TLR/IL-1R signalling remains controversial. Initial functional studies suggest that Pellino1 is involved in the Lys-63 polyubiquitination of IRAK molecules in response to IL-1 stimulation and that this was required for NF-κB activation (Jiang, Johnson et al. 2003), however more recently this was contradicted by the generation of the

Pellino1$^{-/-}$ mouse that showed Pellino1 was dispensable for IL-1 signalling and was involved in TLR3/4 signalling via TRIF (Chang, Jin et al. 2009), which may reflect the differences between the human and the mouse. Available data on Pellino1 function is not only contradictory but also relies mainly on overexpression studies, which may not reveal the true physiological roles and limited experiments have been carried out on the human. To investigate the role of Pellino1 in human cells, a Pellino1 stable knockdown was generated in the HeLa cell-line.

The stable Pellino1 knockdown was generated in HeLa cells using the same method as the MyD88 knockdown in BEAS-2B. A Pellino1 targeting shRNA was packaged into a lentivirus and used to transduce HeLa cells and cells that had incorporated the plasmid were selected and single-cell cloned. Three clones (clones 8, 9 and 12) exhibited Pellino1 knockdown by RT-PCR and qPCR. Clone 12 showed a marked reduction in Pellino1 mRNA levels and was used to test the functional role of Pellino1 in human HeLa cells.

HeLa clone 12 exhibited stable Pellino1 mRNA knockdown throughout the functional experiment. This Pellino1 knockdown led to a slight increase in IL-8 generation in response to both IL-1β and poly(I:C) stimulation, however TNFα responses were unaffected by Pellino1 knockdown. This increase in IL-8 generation may suggest that Pellino1 is playing a negative regulatory role in the TLR/IL-1R signalling pathway. Another member of the Pellino family, Pellino3b, has been shown to be a negative regulator of IL-1 signalling possibly by sequestering of IRAK1 at the membrane (Xiao, Qian et al. 2008). However, these data contradict other previous functional studies on Pellino1 that suggest it acts as an activator of both IL-1 signalling by targeting IRAK molecules (Jiang, Johnson et al. 2003) and TLR3 signalling by targeting RIP1 (Chang, Jin et al. 2009).

Very recently, Pellino1 has been shown to be a negative regulator of T cell activation and prevents autoimmunity. T cells have a high abundance of Pellino1 (but not Pellino2 or Pellino3) and Pellino1$^{-/-}$ T cells were hypersensitive to activation with anti-CD3 and anti-CD28 antibodies and had a higher proportion of memory T cells (CD62L^{+}) in older Pellino1$^{-/-}$ mice. Abnormal activation of T cells is often associated with autoimmune diseases with the breakdown of T cell tolerance of self tissues. Pellino1$^{-/-}$ mice spontaneously develop systemic autoimmunity and have enlarged lymph nodes as early as 10 weeks of age. Older Pellino1$^{-/-}$ mice have infiltrating immune cells in many organs including the kidney, liver and lung. This autoimmunity is thought to be caused by the lack of negative regulation of the late-phase NF-κB transcription factor, c-Rel, by Pellino1. Pellino1 is thought to be a negative

regulator of T cell activation by the attachment of Lys-48 polyubiquitin chains to c-Rel (Chang, Jin et al. 2011). These data suggest that Pellino1 is capable of acting as a negative regulator of inflammation in certain cell-types and therefore may be involved in the negative regulation of TLR/IL-1R signalling in HeLa cells.

However, the increase in IL-8 in Pellino1 knockdown HeLa clone 12 was not significant and could be a non-specific effect of the transduction process (**see section 6.6**) leading to the conclusion that Pellino1 knockdown in HeLa has little effect on IL-8 production in response to TLR/IL-1R agonists. This may be due to HeLa cells utilising different signalling pathways or regulatory proteins in response to poly(I:C) that are independent of Pellino1.

It is also important to note that HeLa cells are an immortalised cell-line that have been cultured in laboratories for over 50 years and bears little resemblance to that of primary cervical epithelial cells and their phenotype can be dramatically altered by changing media conditions (Masters 2002). HeLa cells are a robust cell-line and are one of the most proliferative cell-lines used in cell culture and due to this robust nature it is possible that Pellino1 is not required for TLR/IL-1R signalling or its role can be performed by another protein in HeLa cells. It is therefore important to recreate these experiments in more physiologically relevant cells.

6.4 BEAS-2B stable Pellino1 knockdown – clone C2 and C12

The functional significance of Pellino1 in human lung airway epithelial cells is so far unknown and this was investigated by the creation of stable Pellino1 knockdown BEAS-2B cell-lines. Pellino1 was knocked down in BEAS-2B using a method that had previously proven successful in both knocking down another target in the same cell-line and knocking Pellino1 down in a different cell-type.

The stable Pellino1 knockdown was created by transducing BEAS-2B cells with a lentivirus carrying a shRNA targeting Pellino1. Cells that had incorporated the plasmid were selected and single-cell cloned. Several clones (clones 2, 3, 4, 5, 10, 12 and 15) exhibited Pellino1 mRNA knockdown when measured with RT-PCR and three clones, clones 2, 12 and 15, were verified using qPCR. The two clones, clones 2 and 12, which exhibited the best Pellino1 mRNA knockdown were used to investigate Pellino1 function in BEAS-2B cells.

Previous data from stable Pellino1 knockdown in HeLa cells showed that IL-8 generation was slightly increased in response to IL-1β and poly(I:C). BEAS-2B cells were used to test whether this phenotype was specific to HeLa cells or is common to other epithelial cell-

lines. It was also hypothesised that the increase in IL-8 generation was due to off-target effects of the transduction process and this issue was addressed by comparing wild-type and Pellino1 stable knockdowns to a stable GFP-expressing BEAS-2B cell-line that was created using the same method as the Pellino1 knockdown lines.

Despite the Pellino1 stable knockdown clones 2 and 12 being from the same batch of cells and initially exhibiting the same degree of Pellino1 mRNA knockdown, the phenotypes each clone exhibited were dramatically different. Clone 12, like HeLa Pellino1 stable knockdown clone 12, exhibited increased IL-8 production in response to IL-1β stimulation compared to wild-type, however produces similar amounts to that of the GFP-expressing cell-line. In contrast, clone 2 produces only marginally more IL-8 in response to IL-1β than wild-type and significantly less than its clone 12 and GFP-expressing counterparts.

The GFP-expressing line produced much higher levels of IL-8 in response to IL-1β compared to wild-type BEAS-2B and these data could suggest that the increase in IL-8 seen in both HeLa and BEAS-2B stable Pellino1 knockdown clones may be a non-specific effect of the transduction process and not a phenotypic difference. It is also important to note that the wild-type BEAS-2B failed to respond to stimuli in all functional experiments.

Similar to results gathered in response to IL-1β, IL-8 production in response to poly(I:C) and TNFα in clone 12 was significantly greater than that of clone 2, wild type and GFP-expressing BEAS-2B cells. This would suggest that clone 12 was more responsive to pro-inflammatory stimuli irrelevant of the intracellular signalling pathways activated and this may be due to selection of a highly responsive cell in the single-cell cloning process. The extremely high production of IL-8 in response to TNFα, which was markedly higher than the GFP-expressing control, suggests that it is not a useful clone to investigate the functional role of Pellino1.

Clone 2 showed produced slightly elevated amounts of IL-8 in response to both poly(I:C) and TNFα compared to wild-type. This non-significant increase may be due to the transduction process as the GFP-expressing control produces the same amount of IL-8 in response to these agonists. In contrast, clone 2 produced much less IL-8 in response to IL-1β compared to the GFP-expressing line, suggesting that it may be playing a role in IL-1β mediated NF-κB activation, which is supported by Jiang et al, 2003 (Jiang, Johnson et al. 2003). This reduction of IL-8 generation in response to IL-1β was not seen in HeLa stable Pellino1 knockdown and this may be due to the fact that HeLa and BEAS-2B cells utilise

different regulatory mediators for IL-1 signalling. Clone 2 IL-8 production is not lower than wild-type production, however in the absence of a working wild-type control, it is not possible to exclude the possibility that clone 2 was successful in showing selective IL-8 reduction in response to IL-1β and poly(I:C).

Stable knockdown clones were routinely analysed by qPCR in order to assess Pellino1 mRNA levels. It was found that Pellino1 expression varied significantly with passage, which is discussed in the following sections. It is for this reason that the data generated from BEAS-2B stable knockdown lines should be interpreted with considerable caution. To corroborate these observations, transient knockdown of Pellino1 was carried out in airway epithelial cells. This approach not only negates the issue of single-cell cloning a hyper/hypo-responsive cell-type but also removes the need to culture cells over long periods, which may allow for re-expression of targeted genes.

6.5 Variable Pellino1 knockdown in stable Pellino1 knockdown BEAS-2B cells

In principle there are two main advantages of stable knockdown lines; the first is the production of a cell-line that can be maintained indefinitely in culture that produces an efficient and constant level of target mRNA knockdown. The second is that these cells do not have to be transfected with siRNA for each experiment and so is time- and cost-effective and reduces the variability of transfection efficiency.

Creation of a stable knockdown cell-line should produce a constant amount of mRNA knockdown, however the three passages used for the functional experiment with the Pellino1 stable knockdown BEAS-2B clones showed a variable amount of knockdown and the level of knockdown generally decreased with passage.

The reduction in the degree of knockdown in Pellino1 stable knockdown BEAS-2B cells could be due to the shRNA being silenced or degraded. This silencing of the Pellino1 targeting shRNA is not seen in HeLa cells as Pellino1 mRNA knockdown was tracked over 8 passages and showed a similar amount of knockdown throughout these passages. The Pellino1 shRNA may be silenced in BEAS-2B cells and not in HeLa cells due to the essential role of Pellino1 in these cells and therefore one would hypothesise that Pellino1 knockdown cells may die in culture, putting a selective pressure on Pellino1 expressing cells. This could be measured in future experiments by measuring the effects of transient

Pellino1 knockdown on growth rates of BEAS-2B cells. Knockdown of Pellino1 in BEAS-2B leads overall to a greater deficiency of the Pellino family as they only express low levels of Pellino3a and the putative negative regulator Pellino3b, whereas Pellino1 knockdown HeLa express significant levels of Pellino2 and Pellino3a. It is important to note that silencing of shRNA does not automatically occur in BEAS-2B cells as the shRNA targeting MyD88 was not subject to silencing or degradation.

One way in which mammalian cells may silence introduced shRNA in later passages is by an antiviral process where foreign RNA is degraded by RNAi. There is increasing evidence to suggest that miRNAs encoded by host cells can regulate viral replication by degrading viral mRNA (Song, Liu et al. 2010) and this process may be activated in response to shRNA that has been transduced into the chromosome. Or the promoter of the shRNA may be silenced by DNA methylation of CpG islands in the 5' regulatory region of the shRNA.

The mechanism in which the Pellino1 shRNA was silenced/degraded in BEAS-2B was not common to all shRNA introduced into the cell as BEAS-2B as MyD88 stable knockdown BEAS-2B cells remained knocked down throughout the functional experiments. The Pellino1 targeting shRNA may be silenced/degraded in BEAS-2B cells due to an essential functional in growth or other cellular processes and is therefore switched off to allow the cells to be maintained in culture. This hypothesis is supported by the slow growth rates of Pellino1 stable knockdown BEAS-2B cells and this phenomenon is present in both stable knockdown clones. However, stable Pellino1 knockdown in HeLa cells did not lead to the silencing/degradation of the shRNA and therefore may lack an essential role for Pellino1.

6.6 Creating stable Pellino1 knockdown in BEAS-2B cells using the Sigma MISSION™ system

Creating a Pellino1 stable knockdown in BEAS-2B cells was unsuccessful despite good mRNA knockdown in the initial passages, as it became clear that knockdown was variable with passage number. Due to the variability in the Pellino1 knockdown clones generated using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System (Invitrogen) it was not possible to investigate Pellino1 function in lung airway epithelial cells in this way and an alternative knockdown system was pursued.

The Sigma MISSION™ system provided four different lentiviruses carrying shRNA targeting Pellino1 and these were used to generate new stable Pellino1 knockdown BEAS-2B cell-lines. The efficacy of each virus was tested in transient knockdown experiments in BEAS-2B

cells and the virus that led to the greatest degree of Pellino1 knockdown was used to generate a stable knockdown clones following single-cell cloning.

This approach was also unsuccessful and none of the clones exhibited Pellino1 knockdown. Surprisingly, many of the clones expressed much greater levels of Pellino1 than wild-type. These data support the hypothesis that Pellino1 shRNA is silenced or degraded over long-term culture of stable knockdown BEAS-2B cells, which could suggest Pellino1 plays an essential role in these cells. Due to the lack of Pellino1 knockdown in these cells, I did not pursue this further.

6.7 General limitations of stable knockdown cell-lines

Historically the investigation of the functional role of cellular proteins has been investigated using over-expression techniques and such, much of the original functional data on the Pellino family has been gathered in this way. More recently however it has become clear that over-expressing a protein does not always reflect its physiological role and many phenotypes are an artefact of the over-expression process. Knockdown or knockout of a protein has been shown to be a more reliable method of exploring functional roles of proteins and this technique is used extensively in cell biology.

One way to produce target mRNA knockdown in cell-lines is by transducing a cell-line with a lentivirus containing a shRNA that can insert into the chromosome and lead to long-term degradation of the target mRNA by RNAi. **Section 4.11** presents data from human cell-line clones that exhibit stable and consistent target mRNA knockdown and the expected phenotype when investigating the highly characterised molecule MyD88. However, there are significant limitations with creating stable knockdown cell-lines and these will be discussed in the following sections.

6.7.1 Cell numbers

Cells that had been transduced with lentivirus were selected for using the antibiotic resistance marker on the plasmid encoding the shRNA. These cells were single-cell cloned to ensure a consistent level of target gene knockdown, however this process also led to variability in the growth of each clone. The dramatic differences in the rate of growth of each clone led to discrepancies in the number of cells in each well of a 24-well culture plate and therefore the overall cytokine outputs could not be compared between wells.

To overcome this problem, cells were seeded at various densities according to their growth rate and cytokine generation was presented as amount of cytokine per 10,000 cells.

However, the seeding densities were an estimate and often slow growing clones would fail to reach confluency. The different degree of confluency could be a reason for the discrepancy in the phenotype in BEAS-2B Pellino1 knockdown clones 2 and 12 as cells that are still in active growth phase may respond differently to those that have senesced in post-exponential growth phases.

6.7.2 What is an appropriate control line?

The phenotype of the Pellino1/MyD88 knockdown cells can only be uncovered with the comparison of a control cell-type in order to measure normal cytokine responses. However, the choice of the correct control cell is cause for controversy. HeLa Pellino1 knockdown clone 12 showed a slight increase in IL-8 generation in response to IL-1β and poly(I:C), however this was not significant and could have been due to off-target effects of the transduction process. To investigate this further, a BEAS-2B GFP-expressing line that was created using the same method as the knockdown clones was included to investigate the function of Pellino1 in BEAS-2B cells. This showed that the GFP-expressing BEAS-2B line produced more IL-8 in response to both IL-1β and poly(I:C), which suggests clones may produce more IL-8 in response to these agonists and therefore wild-type cells that have not undergone single-cell cloning may not be a representative control line.

It is important to note that even GFP-expressing cells would not be the ideal control cell as they do not undergo the RNAi process. If this was to be repeated, a single-cell cloned cell-line could be created that had been transduced with a non-targeting shRNA to offer a relevant control cell-line.

6.7.3 Cell-type limitations

The creation and verification of a stable target knockdown cell-line occurs over several months and the length of the protocol limits the range of cells that can be transduced. Cell-lines are ideal for this purpose as the physiology of the cell should not change over culture time and can be maintained indefinitely in culture. However, these cells are not always representative of normal human tissues as they are often derived from cancer cells or have been immortalised by transfection of viral genes that inhibit key molecules involved in cell death such as p53 and retinoblastoma.

Certain primary human cells, such as epithelial cells, endothelial cells, fibroblasts, myocytes, smooth muscle cells and osteoblasts are able to be cultured in tissue culture conditions, however these cells can only be maintained for a small number of passages (approximately 6 for primary bronchial epithelial cells) due to the senescence of these cells

and this passage restriction makes them unsuitable for the creation of stable knockdowns by lentiviral vector.

6.7.4 Heterogeneity of cell populations

Stable knockdown cell-lines were created by transduction of targeted shRNA and cells that had incorporated the plasmid encoding the shRNA were selected for using the antibiotic resistance marker. Antibiotic resistant cells were single-cell cloned to ensure a consistent target gene knockdown, however this led to the introduction of morphological and physiological changes between different clones. Cell-lines are naturally a heterogeneous population of cells, each of which grow at slightly different rates and respond slightly differently to pro-inflammatory agonists and therefore the single-cell cloning process could select a clone with a predisposed phenotype that is independent of the target gene knockdown. This is a possible reason why the BEAS-2B Pellino1 stable knockdown clones 2 and 12 produce considerably different amount of IL-8 in response to IL-1β, poly(I:C) and TNFα, despite exhibiting similar levels of Pellino1 mRNA knockdown.

As discussed in **section 6.7.2**, the choice of the correct control cell is difficult for the comparison with the functional responses of stable knockdown cells. If this experiment was to be repeated then it may be beneficial to forgo the single-cell cloning process and use a heterogeneous population of knockdown cells as this would make them more comparable with wild-type cells and would keep growth rates similar. If a heterogeneous population of knockdown cells was used it would be important to measure the level of target knockdown after each functional experiment to ensure consistency.

6.7.5 Cytotoxicity/off-target effects of shRNA

Previous studies have shown that the stable introduction of shRNA leads to cell toxicity and off-target effects due to overloading of the microRNA (miRNA) pathway leading to dysregulation of unrelated genes (Grimm, Streetz et al. 2006; McBride, Boudreau et al. 2008; Beer, Bellovin et al. 2010). These observations could suggest that there is a selective pressure for cells that do not express shRNA and due to the removal of antibiotic after the initial single-cell cloning process, the selective pressure of the antibiotic resistance is removed. These data also calls into question the usefulness of stable shRNA knockdown cell-lines due to the off-target effects caused by the saturation of the miRNA pathway leading to expression of genes that are usually down-regulated under physiological conditions.

6.8 Tet Repressor line

Many of the limitations of using stable knockdown cell-lines are due to the lack of relevant control cells that have undergone the same transduction process. Many of these limitations can be overcome by creating a Tet repressor line. A Tet repressor line is created using a similar method used to create stable knockdown cell-lines where a lentivirus carrying a plasmid encoding the Tet repressor protein was used to transduce BEAS-2B cells and the cells that had incorporated the plasmid into their genomes were selected for using an antibiotic resistance marker. These cells would constitutively express the Tet repressor protein that binds to the H1/TO promoter used to drive transcription of shRNA that are created using the BLOCK-iT^{TM} Inducible H1 RNAi system. When Tet repressor protein is bound to the H1/TO promoter it blocks transcription of the shRNA of interest. Upon addition, tetracycline binds to the Tet repressor protein and changes its conformation so it can no longer bind to the H1/TO promoter and therefore the shRNA is transcribed leading to gene knockdown. Thus, this method allows for the inducible regulation of gene knockdown and would therefore overcome problems associated with essential genes that are required for growth or fundamental cellular functions as discussed in **section 6.4.**

Inducible gene knockdown by the Tet repressor protein allows for the same cells to be used as the control cells that have been through the same transduction process and are the same clone. This is beneficial as it will remove any variability in cytokine responses and growth rates and the only difference between the control and knockdown cells is the addition of tetracycline. This method provides an efficient way to ensure that the phenotype of the clone is due to target gene knockdown.

Attempts to clone Tet repressor expressing HeLa and BEAS-2B lines were unsuccessful as no cells exhibited antibiotic resistance. The transient transduction experiment showed that the plasmids and lentivirus were capable of transducing cells. The antibiotic kill-curve experiment was repeated and ensured the correct amount of blasticidin was used to select for transduced cells and finally the presence of the antibiotic resistance gene was verified by RT-PCR. All of these data indicated that there was a mutation in the blasticidin resistance gene that rendered it inactive. In order to take this work forward a new lentivirus with a sequence-verified plasmid carrying the Tet repressor would have to be made.

6.9 Transient Pellino1 knockdown in HeLa cells

Stable knockdown of Pellino1 in HeLa cells was successful and provided a clone that exhibited consistent Pellino1 knockdown. This stable Pellino1 knockdown clone showed a slight increase of IL-8 production in response to IL-1β and poly(I:C) stimulation, however this was compared to a heterogeneous population of wild-type HeLa cells and this slight increase may be due to off-target effects. This hypothesis was tested by using siRNA to transiently knockdown Pellino1 and comparing cytokine responses to that of cells that had been transfected with a non-targeting scrambled siRNA.

HeLa cells that had been transfected with Pellino1 targeting siRNA had significantly reduced Pellino1 mRNA compared to mock and scrambled control cells. Pellino1 knockdown HeLa cells showed preserved IL-8 generation in response to IL-1β and TNFα and a slight reduction in IL-8 and RANTES production in response to poly(I:C). These data do not recapitulate the findings from the stable Pellino1 knockdown in HeLa cells and suggests that wild-type HeLa are not an adequate control cell in stable knockdown line experiment and stable cell-lines may not be the most effective way of exploring functional roles of signalling molecules for reasons outlined in **section 6.6**. Interestingly, transient Pellino1 knockdown in HeLa cells contradicts original functional data on Pellino1 that links it to IL-1 mediated NF-κB activation (Jiang, Johnson et al. 2003) and supports more recent data from the Pellino1$^{-/-}$ mouse that shows that Pellino1 is dispensable in IL-1 signalling (Chang, Jin et al. 2009).

It is important to note, however, that HeLa cells do not produce a large amount of IL-8 or RANTES in response to poly(I:C) (within the 0-500 pg/ml range) and therefore these cells may not express very many TLR3 receptors and therefore this slight reduction may only be a minor shift in the baseline. It was important to carry out these experiments in an easily transfectable human cell-line such as HeLa so that functional data could be compared with that of Pellino1 stable knockdown in lung airway epithelial cells.

6.10 Transient Pellino1 knockdown in airway epithelial cells

To overcome the limitations of stable knockdowns, the function of Pellino1 in human airway epithelial cells was investigated by transiently knocking down Pellino1 in BEAS-2B and PBECs using a targeted siRNA. Cytokine responses in transient Pellino1 knockdown cells were compared to that of cells that had been transfected with a non-targeting scrambled siRNA.

6.10.1 Effect of transient Pellino1 knockdown in response to TLR/IL-1R agonists in airway epithelial cells

Epithelial cells were transfected with siRNA targeting Pellino1 or a non-targeting scrambled siRNA and these cells were stimulated with TLR/IL-1R agonists to investigate the effect of Pellino1 knockdown in airway epithelium. Transfection of Pellino1 targeted siRNA led to the significant reduction of Pellino1 mRNA compared to mock and scrambled transfected cells.

Stimulation of Pellino1 knockdown BEAS-2B cells and PBECs with poly(I:C) led to a significant reduction of IL-8 generation, which supports data from Chang *et al* who show a significant reduction in blood concentrations of other pro-inflammatory cytokines, IL-6 and TNFα, in response to poly(I:C) in Pellino1$^{-/-}$ mice (Chang, Jin et al. 2009). These data suggest that Pellino1 may be an important mediator of the TLR3 signalling pathway.

Original functional studies on Pellino1 suggest that it functions in the IL-1 signalling pathway (Jiang, Johnson et al. 2003) and this is supported by data from Pellino1 knockdown BEAS-2B cells showing a significant reduction in IL-8 production in response to IL-1β stimulation. However, these data are gathered from immortalised cell-lines, which despite being a useful model for exploring protein function in human cells, these cells are not always truly representative of primary human cells.

In contrast to BEAS-2B cells, Pellino1 knockdown PBECs show preserved IL-8 generation in response to IL-1β stimulation, which is supported by studies using the Pellino1$^{-/-}$ mouse (Chang, Jin et al. 2009). It is possible that the reason for the controversy of the involvement of Pellino1 in the IL-1 response may be due to the effectiveness of each cell-type to respond to the agonist. PBECs produce almost 10-fold less IL-8 in response to IL-1β stimulation compared to BEAS-2B cells. It is conceivable that because PBECs are less responsive to IL-1β, they may require fewer signalling control points and therefore Pellino1 may not be required for this pathway. A reduction in IL-8 in response to IL-1β may only be observed in cell-types that produce a threshold amount of cytokine in response to IL-1 and therefore may require extra signalling control mechanisms. However, HeLa cells produce similar amounts of IL-8 in response to IL-1β as BEAS-2B cells and Pellino1 knockdown has no effect on this production suggesting that Pellino1 involvement in the IL-1 signalling pathway is also cell-type specific perhaps relating to the repertoire of Pellino proteins that are expressed.

As IL-8 was profoundly reduced in response to poly(I:C), it was hypothesised that other NF-κB-regulated cytokines would also be reduced in response to poly(I:C) in Pellino1 knockdown PBECs, however IL-6 production was only slightly reduced and these data were not statistically significant. This reduction of IL-6 was also seen with IL-1β and TNFα treatment, although PBECs only produced a small amount of IL-6 in response to these agonists.

Pellino1 seems to play specific roles in the production of cytokines in response to poly(I:C). To investigate the role of Pellino1 in the production in a range of different cytokines a cytometric bead array was used to measure cytokine release in poly(I:C) stimulated Pellino1 knockdown PBECs. The array data discovered Pellino1 played specific roles in cytokine release, where Pellino1 knockdown cells produced less TNFα and GM-CSF and to a lesser extent MCP-1, MIP-1α and MIP-1β. TNFα, like IL-8 and IL-6, is a NF-κB regulated pro-inflammatory cytokine and GM-CSF is involved in the survival and differentiation of neutrophils. MCP-1, MIP-1α and MIP-1β are all chemokines that are primarily involved in the recruitment of monocytes. These data suggest that Pellino1 would be a good target to down-regulate leukocyte-mediated in pro-inflammatory environments such as the airways of patients with asthma or COPD. However, these data also reveals a complex role for Pellino1 where it is important in the production of a specific subset of cytokines, since the production of other NF-κB regulated cytokines including IL-1α and IL-1β and the anti-inflammatory cytokine IL-10 was completely unaffected by Pellino1 knockdown.

The interferon-stimulated gene RANTES was unaffected in Pellino1 knockdown BEAS-2B and PBECs. These data suggest that Pellino1 may act as an activator of NF-κB in the TLR3 signalling pathway but is independent of TLR3-mediated IRF activation, however recent data from Smith *et al* who show that Pellino1 E3 ligase activity is enhanced in response to poly(I:C) by phosphorylation by the IKKε/TBK1 kinases (Smith, Liu et al. 2011) and is therefore Pellino1 activation may be intrinsically linked to IRF activation even if it does not directly regulate it.

Interestingly however, another interferon-stimulated gene, IP-10, was significantly reduced in Pellino1 knockdown PBECs, but only with high doses of poly(I:C) (25 μg/ml). This supports data from Smith *et al* that suggest Pellino1 plays a role in IRF activation in response to TLR3 activation (Smith, Liu et al. 2011). It is possible that Pellino1 is only required for IP-10 production when the cells are under stress through high concentrations of dsRNA. Or this phenomenon may reflect the lack of other stimuli from other cytokines

such as IL-8 in Pellino1 knockdown cells. It is important to note that these data suggest that overall, the activation of IRF-stimulated genes are preserved under more physiologically relevant concentrations of poly(I:C) (0.1 – 10 μg/ml), which implies that Pellino1 selectively facilitates NF-κB signalling.

Many studies have shown that the airways of asthma patients suffer from chronic inflammation and hyper-responsiveness and viral infections, particularly rhinoviral infections, are a common cause of asthma exacerbations (Johnston, Pattemore et al. 1996; Gern and Busse 1999; Pauwels, Pedersen et al. 2003). Considering this, we wanted to investigate the role of Pellino1 in epithelial cells isolated from asthmatic patients. These data were collected in collaboration with Irene H Heijink at the University Medical Center in Groningen. Pilot data from Pellino1 knockdown in asthmatic PBECs confirmed our data from normal PBECs where stimulation with poly(I:C) led to a reduction in IL-8 generation and IL-1 responses and RANTES production were both unaffected.

It is important to note, however, that the baseline levels of IL-8 in asthmatic PBECs were approximately 10 times higher than amounts measured in normal PBECs. This finding may reflect the proinflammatory environment of the asthmatic airway. This increase in baseline masks the dose-dependent response to IL-1β as PBECs (asthmatic or normal) do not respond highly to this agonist and therefore the signal is saturated in asthmatic PBECs. Also, this experiment has only been carried out on one donor thus far and therefore responses to Pellino1 knockdown may differ in different patients who exhibit different severities of the disease.

It has become more apparent that the role of Pellino1 is as diverse as the expression pattern of the Pellino family in different cell types. It is likely that the role of Pellino1 is linked to this expression pattern and also the responsiveness of each cell-type to specific agonists. The role of Pellino1 is further complicated through possible feed-back mechanisms that only uncovers a phenotype at high doses of stimulus. Despite this complex role, these data suggest that Pellino1 is important in TLR3-mediated NF-κB activation and supports other work carried out in primary murine cells (Chang, Jin et al. 2009).

6.10.2 Effect of transient Pellino1 knockdown in response to RV1B infection in airway epithelial cells

Pellino1 was shown to play a possible role in TLR3 signalling in mediating NF-κB activation but seemed to be independent of IRF activation in airway epithelial cells. TLR3 is

responsible for detecting viral particles within the cell by binding to double stranded RNA, poly(I:C) is a synthetic double stranded RNA and TLR3 agonist, however does not recapitulate the full phenotype of viral infection as it does not replicate within the cell. We wanted to investigate the role of Pellino1 in response to a natural human viral pathogen as, unlike with poly(I:C) stimulation, infection with a natural virus would lead to the physiological activation of receptors and viral signalling pathways that may not occur in the absence of replicating virus.

The role of Pellino1 in response to human pathogens is yet to be described and therefore the effect of Pellino1 knockdown on cytokine release in response to a natural viral pathogen was investigated. Human epithelial cells were transiently transfected with siRNA targeting Pellino1 and these cells were infected with RV1B.

Pellino1 knockdown in PBECs infected with RV1B led to the significant reduction in the NF-κB regulated cytokines IL-8 and IL-6, whereas the IRF-stimulated genes RANTES and IP-10 were unaffected. These data indicate that Pellino1 is important for the activation of inflammatory signalling in response to viral pathogens and that it functions in the NF-κB branch of the TLR3 signalling pathway, selectively. In contrast, RV1B infection in Pellino1 knockdown BEAS-2B cells had no effect on IL-8 production, which may represent differences in immortalised and primary cells and other viral detection mechanisms such as the cytoplasmic receptors RIG-I/MDA5 may play an overriding role in the detection of RV1B in these cells.

Pellino1 has previously been shown to function in the TLR3 signalling pathway as the Pellino1$^{-/-}$ mouse shows significant reduction of IL-6 in response to poly(I:C) (Chang, Jin et al. 2009), however this is the first description of the role of Pellino1 in response to a natural viral pathogen. In contrast to PBECs, Pellino1 knockdown in BEAS-2B cells had no effect on IL-8 generation and this may reflect fundamental differences in immortalised and primary cells. The TLR3 phenotype in PBECs is more prominent than in BEAS-2B cells as Pellino1 knockdown BEAS-2B cells still produce ~40ng/ml IL-8 in response to poly(I:C), whereas Pellino1 knockdown PBECs only produce ~10 ng/ml IL-8 where both scrambled control transfected cell types produce ~80 ng/ml IL-8. These data suggest that BEAS-2B cells are able to signal more effectively through TLR3 in response to poly(I:C) in the absence of Pellino1 than PBECs, however the role of Pellino1 in primary airway cells seems to have an essential role in response to viral infection.

Pellino1 knockdown in RV1B infected PBECs has no effect on the IFN-stimulated genes RANTES and IP-10 or production of the type I or III IFNs suggesting Pellino1 is not involved in IRF activation, which is interesting as a very recent study has shown that Pellino1 binds to and is phosphorylated by IKKε/TBK1, which are key mediators in the IRF signalling pathway (Smith, Liu et al. 2011). However, this study did not examine cytokine generation and another study has showed there is no significant difference in IFN-β release in the blood of poly(I:C) stimulated Pellino1$^{-/-}$ mice (Chang, Jin et al. 2009). An interesting finding from a recent study suggests that IFN production may even be independent of TLR3, as RV1B infected TLR3$^{-/-}$ mouse produce the same amount of type I and type III IFNs in the lung as wild-type mice (Wang, Miller et al. 2011).

A major difference between poly(I:C) stimulation and infection with RV1B is that the viral pathogen replicates within the cytoplasm of hosts cells. Considering this, it is also important to consider the retinoic acid-inducible receptors (RLRs) MDA5 and RIG-I. These cytoplasmic viral receptors have also recently been shown to signal through RIP1 to activate both the NF-κB and IRF3 signalling pathway (Yu and Levine 2011), however Pellino1 may selectively activate the NF-κB or MAPK pathway in the early phase response as it has been shown that TLR3 responses are dominant during this phase and are responsible for the subsequent upregulation of MDA5 and RIG-I receptors (Slater, Bartlett et al. 2010). Thus, if the Pellino1 target is RIP1 as it has previously been suggested (Chang, Jin et al. 2009) and is investigated in this thesis, then these data suggest that the role of Pellino1 is important in the early phase response to Rhinovirus where RIP1 is involved in TLR3 mediated NF-κB activation via the recruitment of TRAF6, TAK1 and the dsRNA-dependent protein kinase PKR (Jiang, Zamanian-Daryoush et al. 2003).

Pellino1 knockdown did not seem to significantly affect viral replication of RV1B in infected PBECs. This result is in keeping with other data showing that the antiviral genes, IFNs and IFN-stimulated genes, were unaffected by Pellino1 knockdown. Production of the type I and III IFNs have potent antiviral activity by upregulating proteins that bind to dsRNA (Williams 1999), inhibit protein translation (Kerr and Brown 1978; Jacobs and Langland 1996), inhibit trafficking of viral ribonucleoprotein complexes (Weber, Haller et al. 2000) and induce apoptosis in virally infected cells (Takizawa, Ohashi et al. 1996; Der, Yang et al. 1997; Balachandran, Kim et al. 1998; Yeung, Chang et al. 1999; Gil and Esteban 2000). As IFN production is preserved in Pellino1 knockdown PBECs, this allows the cell to clear the infection as efficiently as control cells. These data suggest that Pellino1 could be a desirable

drug target for the treatment of rhinoviral-induced asthma exacerbations as patients could overcome the initial infection without creating a neutrophilic inflammatory environment leading to hyper-responsiveness of the airways. These data suggest that Pellino1 knockdown may even be beneficial to antiviral immunity. However, this could be an off target effect of the down-regulation of other NF-κB regulated cytokines that allows the infected cells to produce slightly more IFN due to the availability of cellular machinery.

Previous work from our group has shown that the MyD88-dependent NF-κB signalling pathway is important for cellular signalling in response to rhinovirus (Stokes, Ismail et al. 2011). These data also suggest that RV1B infection activates the IL-1R signalling pathway by autocrine IL-1β. Considering these data, it is possible that Pellino1 is involved in ubiquitinating IRAK-1 in the IL-1R signalling pathway in response to rhinoviral infection. However, this study showed RV1B replication to be significantly increased in MyD88 knockdown airway epithelial cells, which is not seen in Pellino1 knockdown cells. Pellino1 has previously shown to bind to and ubiquitinate RIP1 in TLR3 signalling, however this has only been described in the mouse in response to poly(I:C) or in overexpression experiments in human cell-lines (Chang, Jin et al. 2009). Following these data, we hypothesised that Pellino1 is involved in the regulation of RIP1 in the early phase response to viral stimulus and therefore knockdown of RIP1 in PBECs would recreate the Pellino1 phenotype in these cells. The Pellino1$^{-/-}$ mouse also suggests that Pellino1 is important for MyD88-independent responses to LPS (Chang, Jin et al. 2011), however human airway epithelial cells do not express much TLR4 and therefore do not respond highly to LPS. These data may suggest that Pellino1 may play role in response to bacterial pathogens, but perhaps only when other cells, such as highly LPS-responsive monocytes, are present in the lung.

6.11 Transient RIP1 knockdown in primary bronchial epithelial cells

The target for Pellino1 E3 ligase activity has been suggested to be RIP1 (Chang, Jin et al. 2009). RIP1 is a multifunctional protein that was originally described as a regulator of apoptosis where the RIP1$^{-/-}$ mouse showed increased rates of apoptosis in both lymphoid and adipose tissue and were highly susceptible to TNFα-induced cell death (Kelliher, Grimm et al. 1998). Interestingly, RIP1 has also been shown to be essential for TLR3-mediated NF-κB activation (Meylan, Burns et al. 2004) and binds directly to TRIF via its RIP homotypic interaction motif (RHIM) where it is then phosphorylated and polyubiquitinated (Cusson-

Hermance, Khurana et al. 2005). The E3 ligase involved in the Lys-63 polyubiquitination of RIP1 in TLR3 signalling remains unclear, however a recent study showed that Pellino1 is able to bind to and ubiquitinate RIP1 upon poly(I:C) stimulation (Chang, Jin et al. 2009). We hypothesised that knockdown of RIP1 would recreate the Pellino1 knockdown phenotype in PBECs and these cells would show a significant reduction in IL-8 production in response to poly(I:C).

To investigate whether RIP1 was the target of Pellino1 E3 ligase activity in human PBECs, RIP1 was knocked down in these cells using targeted siRNA, following which they were stimulated with TLR/IL-1R agonists. As in Pellino1 knockdown, RIP1 knockdown PBECs showed preserved IL-1 and TNFα signalling. The latter was surprising since RIP1 has previously been shown to be an essential mediator of TNFR signalling. For example, in RIP-deficient Jurkat cells, TNFα mediated NF-κB activation is abolished (Blonska, You et al. 2004) and the $RIP1^{-/-}$ mouse fails to activate NF-κB in response to TNFα (Kelliher, Grimm et al. 1998). This phenotype is not represented in PBECs, suggesting a possible alternative mechanism of IL-8 production in response to TNFα in these cells.

Pellino1 knockdown in PBECs led to the significant reduction of IL-8 in response to poly(I:C), however conversely in RIP1 knockdown PBECs IL-8 was significantly increased, suggesting that RIP1 is a negative regulator of TLR3 signalling. This is in contrast to data from the mouse which identifies RIP1 as an essential activator of NF-κB in response to poly(I:C) (Meylan, Burns et al. 2004). A recent study however has shown RIP1 to be a negative regulator in non-canonical NF-κB signalling in response to TNFα, where RIP1 is involved in the degradation of NIK by the stabilisation of TRAF2 (Kim, Morgan et al. 2011). Another study has also shown RIP1 fragments to play an inhibitory role in viral signalling. This occurs by the recruitment of RIP1 and caspase-8 to the activated cytoplasmic viral receptor RIG-I, which leads to cleavage of RIP1 by caspase-8 and these cleavage products inhibit IRF activation (Rajput, Kovalenko et al. 2011). These studies support a negative regulatory role in TLR signalling.

However, despite this negative regulatory role of RIP1 being suggested by significant increase in IL-8 generation, it is important to note that IL-6 production is unaffected by RIP1 knockdown. This may reflect the complex regulatory mechanisms that control expression of this pleiotropic cytokine as IL-6 has been shown to act as both a pro- and anti-inflammatory cytokine and was initially described as an antiviral cytokine that is upregulated in response to IFNγ (Heinrich, Behrmann et al. 1998). Interestingly, IL-6 in both

Pellino1 and RIP1 knockdown PBECs is largely unaffected in response to poly(I:C), however it is significantly reduced in Pellino1 knockdown cells infected with RV1B, therefore RIP1 may be utilised as a negative regulator for IL-6 production in signalling pathways activated by natural viral pathogens. I would hypothesise that IL-6 would be significantly increased in response to RV1B infection in RIP1 knockdown PBECs.

Although the data from this thesis suggest that Pellino1 positively regulates TLR signalling, Pellino proteins have also been shown to play a negative regulatory role in NF-κB activation (Xiao, Qian et al. 2008).Very recent data from Chang *et al* have shown that Pellino1 plays a negative regulatory role in the activation of T cells and thus has a protective role against autoimmunity. This negative regulatory role is accomplished by the introduction of Lys-48 polyubiquitin chains onto the NF-κB protein c-Rel by Pellino1, which targets c-Rel for degradation thus restricting T cell activation (Chang, Jin et al. 2011). A possible, and that this stage hypothetical, mechanism for Pellino1 action in TLR3 signalling is that it could be a negative regulator of the inhibitory role of RIP1. Therefore in resting airway epithelial cells, RIP1 is constitutively expressed to suppress TLR3 signalling, however upon viral stimulation Pellino1 facilitates the addition of Lys-48 polyubiquitin chains onto RIP1, leading to its degradation. It would follow that with RIP1 knockdown there would be a significant increase of IL-8 due to the lack of negative regulation and with Pellino1 knockdown there would be no degradation of RIP1, which in turn would lead to the accumulation of the negative regulator causing a significant reduction in IL-8. Further work into the action of RIP1 in airway epithelial cells, Pellino1 targeting of RIP1 and the type of ubiquitination that RIP1 undergoes by Pellino1 would need to be examined to investigate this proposed mechanism. A summary of this mechanism can be found in **Table 1**.

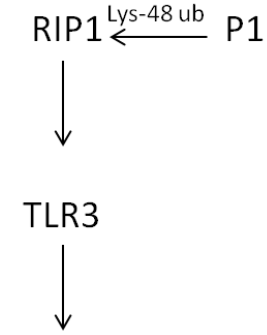
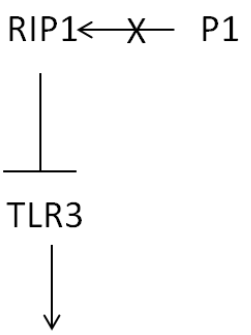
Control	+ viral stimulus		
	Control	RIP1 knockdown	Pellino1 knockdown
			

Table 6.1: Possible mechanism for the negative regulation of RIP1 by Pellino1

This table outlines a hypothetical mechanism for the negative regulation of RIP1 by Pellino1 in PBECs. Under resting conditions, TLR3 is inactive and RIP1 acts as a negative regulator of TLR3 signalling. Upon viral stimulus, RIP1 is degraded by the addition of Lys-48 ubiquitin chains by Pellino1 and this allows downstream signalling to occur leading to IL-8 production. In RIP1 knockdown PBECs, the negative regulation of TLR signalling is removed, leading to a significant increase in IL-8 production in response to viral stimulus. However, in Pellino1 knockdown cells the negative regulation of RIP1 is removed leading to a build up of the negative regulator of TLR3 signalling leading to the significant decrease of IL-8 in response to viral stimulus.

6.12 Limitations of transient knockdown using transient siRNA knockdown

Using siRNA to knockdown target genes has been highly successful in a variety of different cell-types including primary human cells. This makes transient siRNA knockdown a desirable tool for knocking down a variety of targets to investigate Pellino1 function further in human airway cells. Despite the success of the experiments performed, there are drawbacks with using siRNA, which are to be discussed in the following sections.

6.12.1 Length of knockdown

The use of siRNA to knockdown target genes only leads to a transient knockdown of the gene of interest and knockdown is only guaranteed for 72 hours post-transfection. This restricts the type of experiment that can be performed as the functional assay needs to be complete within the timeframe of the siRNA knockdown. A chronic model of inflammation that would be useful for investigating the responses of diseased airway epithelium could not be used for transient gene knockdown. Also, if the proteins is stable over a long period of time and has a low turnover rate then the effect at the mRNA level may not affect the protein level.

6.12.2 Transfection efficiency

As the length of knockdown is transient, each experiment must be transfected with the siRNA and therefore the transfection efficiency may vary between experiments. If the transfection efficiency of the siRNA is variable then the level of knockdown may differ between experiments. To control for this variation, the knockdown efficiency needs to be tested in every experiment performed.

6.12.3 Time and cost implications

Due to the length of knockdown, every experiment needs to be transfected with siRNA and efficiency of the knockdown needs to be verified by qPCR or western blot. This, along with using multiple agonists with a dose response, creates a large demand for siRNA and control siRNA and verification tools such as antibodies or qPCR reagents. For these reasons, siRNA knockdown of target genes is a time-consuming and expensive process compared to that of making a stable knockdown cell-line.

6.12.4 Effect of transfection process/reagents

As functional experiments need to be performed when there is efficient gene knockdown, cells are often stimulated 24 hours after transfection. Unlike stable knockdowns, transient

knockdown cells have only had approximately 18 hours to recover from the transfection process and addition of the transfection reagent. This may make cells more sensitive to pro-inflammatory agonists, and may be primed for TLR activation due to the introduction of siRNA. Using a scrambled non-targeting siRNA as a control helps to distinguish any effects of the transfection process. However, phenotypes may be perturbed by the transfection process.

6.12.5 Protein turnover

We, and others, found that the two commercially available antibodies from Abcam and Santa Cruz were not reliable tools, which makes verification of Pellino1 knockdown at the protein level difficult. Some proteins, especially scaffolding proteins involved in cell signalling, are incredibly stable and therefore protein turnover is very slow. As the siRNA is thought to be degraded within 3 days of transfection, it may be that the turnover of the protein means that despite having significant knockdown at the mRNA level, the level of target protein remains constant.

6.12.6 Toxicity of transfection reagent

There are many different lipid-based transfection reagents that are commercially available. Many of which are efficient at leading to gene knockdown, including Metafectene and Dharmafect 1-4. However, these transfection reagents were extremely toxic to the cells and would lead to approximately 60% cell death. Different cell types may require different transfection reagents, however in lung airway epithelial cells Lipofectamine is capable of producing efficient knockdown without being toxic to the cells.

6.13 Canonical and non-canonical NF-κB activation in response to poly(I:C) in epithelial cells

It is well documented that activation of IL-1R and TLRs, including TLR3, leads to the activation of NF-κB. Canonical NF-κB proteins, p65, RelB and c-Rel, are activated upon the degradation of the inhibitory proteins, IκBα and IκBβ, which releases active NF-κB dimers that can translocate to the nucleus and induce the transcription of target genes. An alternative arm of signalling exists, which is known as the non-canonical NF-κB pathway. The non-canonical NF-κB proteins, p100 and p105, are produced as precursor proteins that contain long C-terminal domains containing ankyrin repeats which keeps the transcription factors in the inactive state in the cytoplasm (Hayden and Ghosh 2004). Upon activation, p100 and p105 are partially degraded to release fragments containing the DNA-binding RHD domains, p50/p52, respectively. These fragments associate with other member of the

NF-κB family, usually RelB, to form active heterodimers that can translocate to the nucleus and activate gene transcription (Hayden and Ghosh 2004).

Degradation of the inhibitory protein IκBα can be used to measure canonical NF-κB activation. In response to IL-1β, BEAS-2B cells exhibit IκBα degradation after 10 minutes of stimulation, which is considered to be a classical degradation timecourse for IκBα. Interestingly, IκBα levels returns to resting levels after 1 hour, however IκBα degradation is slightly delayed in response to poly(I:C) where IκBα is degraded after 45 minutes of stimulation and returns to resting levels after 4 hours. It is likely that the difference in IκBα degradation kinetics between IL-1β and poly(I:C) is due to the location of the cognate receptors as IL-1R is located on the cell surface whereas poly(I:C) has to cross the membrane to activate TLR3. Despite the time differences, these data suggest that both IL-1β and poly(I:C) stimulation of BEAS-2B cells leads to the activation of canonical NF-κB signalling.

Similarly to BEAS-2B cells, PBECs stimulated with IL-1β results in IκBα degradation after 10 minutes, following which IκBα levels return to normal by 45 minutes, which suggests that PBECs are capable of activating the canonical NF-κB signalling pathway. Conversely, IκBα degradation in response to poly(I:C) could not be detected in PBECs at any timepoint tested. Timecourses of 10-240 minutes and 2-24 hours were performed and no evidence of canonical NF-κB activation (via IκBα degradation) could be measured in these cells. Alternative transcription factors that are involved in viral signalling in PBECs were investigated, including the activation of p38, an important MAPK that is involved in the activation of an independent transcription factor AP-1. In IL-1β stimulated PBECs there was a measurable increase in p38 phosphorylation after 15 minutes of stimulation which was reduced to below that of resting cells after 1 hour of stimulation. In contrast, phosphorylation of p38 MAPK could not be visualised in response to poly(I:C) for any of the timepoints tested.

As PBECs produced a large amount of IL-8 in response to poly(I:C) but did not seem to utilise canonical transcription factors, the use of the non-canonical signalling pathway was explored in these cells. Non-canonical NF-κB activation is a process where a precursor protein p100 is phosphorylated and cleaved to produce an active p52 fragment that dimerises with either RelB, c-Rel or p65 which can translocate to the nucleus and active target gene transcription (Hayden and Ghosh 2004). After 16 hours and most predominantly after 24 hours of poly(I:C) stimulation, there is an increase of the p52 active

fragment and also a slight increase in the p100 precursor protein. These data suggest that the non-canonical NF-κB signalling may be involved in TLR3 signalling in PBECs. It also offers a reason for the differences in poly(I:C)-induced IL-8 production between BEAS-2B cells and PBECs as Pellino1 knockdown in the former cell type produces approximately four times as much IL-8 than in PBECs, which may reflect the efficiency/timecourse of signalling through the two signalling pathways. It is possible that poly(I:C) leads to the activation of both canonical and non-canonical NF-κB activation in BEAS-2B cells, however Pellino1 may be selective for the non-canonical signalling pathway and thus Pellino1 knockdown only reduces IL-8 production that is dependent on p100 processing. In contrast, PBECs seem to predominantly utilise non-canonical signalling and therefore the reduction in IL-8 in Pellino1 knockdown cells is more pronounced.

Very preliminary data may suggest that Pellino1 plays a role in p100 processing in PBECs in response to viral stimulus as the increase of p100 and p52 in poly(I:C) stimulated Pellino1 knockdown PBECs was not as prominent as in the scrambled control. However this finding needs to be verified with further experiments. These data in conjunction other published studies led to a possible hypothetical mechanism of action for Pellino1 where in poly(I:C) stimulated PBECs, TLR3 activation leads to the degradation of the negative regulator of non-canonical signalling RIP1 via the addition of Lys-48 ubiquitin chains by Pellino1. RIP1 has previously been shown to be a negative regulator of non-canonical NF-κB activation in response to TNFα (Kim, Morgan et al. 2011) and Pellino1 has been shown to be negative regulator of T cell activation by mediating the addition of Lys-48 of the NF-κB protein c-Rel (Chang, Jin et al. 2011). The negative regulatory roles of these proteins have not previously been described in the TLR3 pathway. The hypothetical mechanism of Pellino1 action is summarised in **Figure 6.1**.

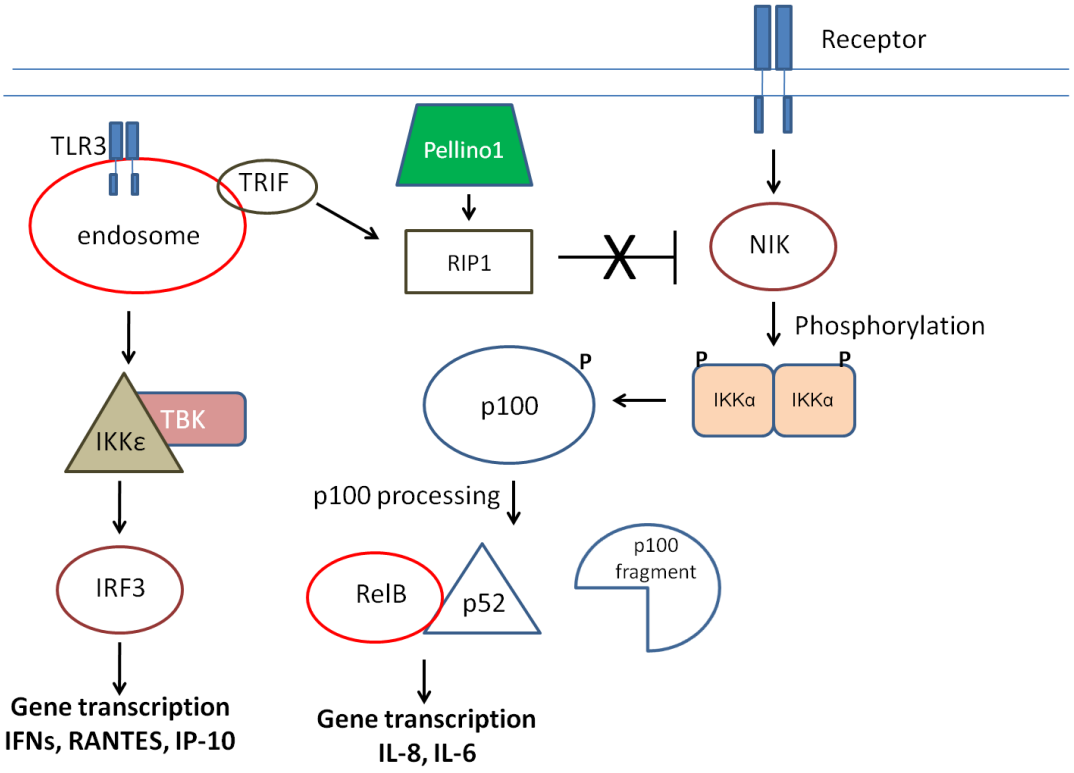


Figure 6.1: The possible role of Pellino1 in TLR3-mediated non-canonical NF-κB activation

Activated TLR3 in response to dsRNA leads to the activation of TRIF. TRIF could directly, or through other as yet uncharacterised mediators, activate Pellino1, which leads to the degradation of the negative regulator, RIP1, and the activation of NIK. NIK phosphorylates IKKα dimers that are responsible for phosphorylating p100, which leads to its partial degradation and release of the p52 active fragment. Active p52 dimerises with RelB, which allows translocation to the nucleus and upregulation of target genes including IL-8 and IL-6. This signalling pathway is independent of IRF3 activation, which leads to the upregulation of IFNs, RANTES and IP-10 and is mediated by IKKε/TBK kinases.

6.14 Limitations of measuring regulation of signalling molecules

Investigating the regulation of signalling molecules is a difficult process due to the transient nature of the event, the magnitude of the potential downstream targets and different kinetics and the different types of modifications that occur to regulate cell signalling.

Regulation of signalling pathways in this thesis have been studied primarily using western blot, which is only a semi-quantitative method and cannot always detect small changes in protein levels and therefore may not be sensitive enough to determine the effect of fine-tuners of signalling such as Pellino1. Also, as signalling events are transient in nature, it is difficult to ensure that the correct timecourse is being used. As such, it is difficult to ensure that IκBα degradation does not occur in PBECs in response to PBECs as the signal is only lost for a maximum of half an hour and therefore could possibly be occurring between timepoints examined.

Signalling networks are complex by nature and involve many different types of regulation. Many proteins are multifunctional and can play different roles in response to different agonists or in different cell types. For example RIP1 has shown to be involved in apoptosis and necrosis (Stanger, Leder et al. 1995); activation of TNFα (Wertz, O'Rourke et al. 2004) and TLR3 signalling (Meylan, Burns et al. 2004); and negative regulation of TNFα-induced non-canonical signalling (Kim, Morgan et al. 2011). Often the multi-functionality of signalling molecules is controlled by posttranslational modifications, as RIP1 undergoes extensive posttranslational modification such as both Lys-63 and Lys-48 polyubiquitination (Wertz, O'Rourke et al. 2004); ubiquitin editing (Wertz, O'Rourke et al. 2004); phosphorylation (Legler, Micheau et al. 2003); and cleavage (Rajput, Kovalenko et al. 2011). Investigating the correct signal at the correct timepoint would be crucial in the understanding of the function of a signalling molecule. A better way of investigating signalling pathways would be to use immunocytochemistry to visualise the cellular location and degradation status of the protein upon stimulation; however these experiments would require a specific antibody.

6.15 Pellino1 as a possible drug target

Diseases of the airway, including asthma and COPD, are among some of the most prevalent diseases in the western world and remain a significant economic and healthcare burden. There are no known cures for these diseases and symptoms are managed using the same treatments that have been in clinic for decades. There is a call for new, more specific

treatments for these diseases and knowledge of the underlying mechanisms of disease is key to achieving this.

Asthma and COPD are inflammatory disorders where sustained inflammation of the airway leads to hyper-responsiveness of the epithelium and is characterised by the prolonged recruitment of inflammatory cells. Asthma exacerbations are a common cause of hospitalisation of asthma patients and viral infections, with rhinoviral infections being the most common, are a major cause of asthma exacerbations. New drug targets are required that do not impair host immunity whilst down-regulating aberrant inflammation. It is for this reason that Pellino1 may prove to be a useful drug target for diseases such as asthma.

Pellino1 knockdown in primary airway cells had no effect on IFN-stimulated genes or IFN production in response to rhinovirus infection, however the pro-inflammatory cytokine IL-6 and the neutrophil chemoattractant IL-8 are both significantly reduced. Thus targeting Pellino1 for the treatment of rhinoviral-induced asthma exacerbations would be beneficial as host cells would retain the antiviral immunity required to clear the infection but would reduce the neutrophilic proinflammatory environment. In addition, previous studies have also shown that impairment of INF-β and IFN-λ in asthmatic patients contributes to Rhinoviral-induced exacerbations (Wark, Johnston et al. 2005; Contoli, Message et al. 2006) showing that the selective nature of knockdown of Pellino1 on inflammatory responses may be crucial for the effective treatment of asthma. Reducing the infiltration of neutrophils in an RV-induced asthma exacerbation by targeting Pellino1 would be beneficial as this would reduce damage to host epithelium caused by excessive production of proteases and reactive oxygen species.

Pellino1 is a desirable drug target due to its selective role in the inflammatory signalling pathways and therefore reduces the risk of off-target detrimental effects on the cell. However, stable knockdown of Pellino1 in BEAS-2B led to a reduction in growth rate and silencing or degradation of Pellino1-targeted shRNA suggesting it may mediate cell growth or essential cellular functions and, if this true this would suggest that Pellino1 would not be an ideal target for long-term antagonism. Preliminary data from Pellino1 knockdown in asthmatic PBECs show that Pellino1 could act as a possible target to down-regulate neutrophilic inflammation whilst retaining antiviral immunity.

There are currently clinical trials underway to target molecules to disrupt ubiquitination. One compound known as Bortezomib (also known as PS-341) is a potent and selective

inhibitor of the 26S proteasome, which disrupts the Lys-48 degradation process. Bortezomib has also been shown to inhibit NF-κB signalling (Jane, Premkumar et al. 2011). This drug has been approved for use in the treatment of cancers such as multiple myeloma and mantle cell lymphoma (Luo, Lin et al.; Chen, Frezza et al. 2011). However, due to the essential nature of the ubiquitin-proteasome pathway in cell homeostasis, the clinical use of Bortezomib is restricted by its dose-limiting toxicity (Chen, Frezza et al. 2011). A more selective and less-toxic therapeutic approach could be achieved by targeting E3 ligases, such as Pellino1, to regulate specific signalling pathways.

A good way to investigate whether blocking Pellino1 in asthmatic lung airway epithelium is beneficial would be to create an epithelial-specific Pellino1 knockout mouse and induce asthma in these mice (Paul, Mishra et al. 2009).

6.16 Future work

6.16.1 NIK transient knockdown

Data from this thesis suggest that poly(I:C) stimulation of PBECs could lead to the activation of the non-canonical NF-κB signalling pathway and that this activation is independent of canonical NF-κB signalling. To investigate this further, NIK, an essential activator of the non-canonical NF-κB signalling pathway, will be transiently knocked down in PBECs and used to measure poly(I:C)-induced IL-8 production. If, as previous data indicates, IL-8 production is reliant on the non-canonical NF-κB pathway then it would follow that there would be a significant decrease in poly(I:C)-induced IL-8 production, recapitulating the Pellino1 knockdown phenotype. Also, to show that this phenotype is independent of canonical NF-κB signalling, p65 will be knocked down in PBECs to show a preservation of poly(I:C)-induced IL-8 production.

6.16.2 Regulation of RIP1

Data from this thesis suggest that RIP1 could be acting as a negative regulator of TLR3 signalling, however this has not previously been shown in other cell-types. RIP1 has been shown to act as a negative regulator of non-canonical signalling in response to TNFα (Kim, Morgan et al. 2011) and therefore may have a predominant role as a negative regulator in cells that only use the non-canonical NF-κB signalling pathway. To investigate the role of RIP1 in TLR3 signalling, regulation of RIP1 in poly(I:C) stimulated PBECs will be visualised using western blot as if it is acting as a negative regulator it is hypothesised that this

protein will be degraded to release the negative regulation and perhaps increased in later timepoints to prevent over-stimulation of the pathway.

6.16.3 Regulation of Pellino1 in response to TLR3 agonists

To date there are no verified commercially available antibodies raised against Pellino1. But very recently, Smith *et al* produced an antibody that is specific for Pellino1 and have shown that Pellino1 is upregulated in response to LPS and poly(I:C) in mouse bone marrow derived macrophages (Smith, Liu et al. 2011). If this, or another Pellino1-specific antibody could be obtained, then the regulation of Pellino1 in primary human cells could be investigated in response to both poly(I:C) and a natural viral pathogen, RV1B.

6.16.4 Production of IL-8 timecourse

It has been proposed that PBECs activate the non-canonical NF-κB signalling pathway in response to TLR3 agonists and that this activation is independent of canonical NF-κB activation. Activation of the non-canonical NF-κB pathway has slower kinetics then that of canonical NF-κB (Beinke and Ley 2004) and increase in p52 active fragments are only visualised after approximately 16 hours of poly(I:C) stimulation in PBECs. To test whether IL-8 production in poly(I:C)-stimulated PBECs is dependent on p100 processing production of IL-8 will be measured every 2 hours over a 24 hour period in PBECs stimulated with poly(I:C) and these data will be compared to that of BEAS-2B cells that have been shown to activate the canonical NF-κB signalling pathway in response to poly(I:C).

6.16.5 Other targets and stimuli

Canonical and non-canonical NF-κB activation has only been studied in PBECs in response to poly(I:C) and therefore canonical NF-κB signalling may be activated upon viral infection of PBECs that is not seen with poly(I:C) due to involvement of the cytoplasmic viral detectors RIG-I/MDA5. However, if IL-8 generation is independent of canonical NF-κB in response to both poly(I:C) and viral infection but is not entirely dependent on p100 processing then the involvement of the alternative NF-κB signalling pathway will be investigated using an antibody that binds both p105 (inactive) and p50 (active) fragments.

It will also be useful to infect Pellino1 knockdown cells with a major group rhinovirus, RV16, as this serotype belongs to the subgroup that makes up ~90% of all rhinoviruses. The effect of infecting Pellino1 knockdown cells with RV16 will be compared to that of RV1B infection. Both RV16 and RV1B will be used to investigate the role of Pellino1 in asthmatic PBECs, which will give more insight into the potential of Pellino1 as a desirable drug target.

Previous data has demonstrated that one way in which TGF-β exhibits its anti-inflammatory effects is by releasing Smad6/7 into the cytoplasm, where they can bind to Pellino1 to inhibit its binding to IRAK1 (Lee, Kim et al. 2010). The effects of TGF-β in response to viral infection and subsequent Smad6/7 activity will be investigated in PBECs.

6.16.6 Pellino1 target and mechanism

The Pellino1 target is still controversial as pervious work has suggested that it binds to IRAK molecules (Jiang, Johnson et al. 2003; Smith, Peggie et al. 2009) or RIP1 (Chang, Jin et al. 2009) however this data is yet to be verified in human airway cells and also the type of ubiquitin chains that are appended by Pellino1 to this target protein will be investigated using ubiquitin-linkage specific antibodies. As data described in this thesis suggests that Pellino1 is playing a key role in the TLR3 pathway and that this pathway activates the non-canonical NF-κB signalling pathway, two possible Pellino1 targets that will be investigated are NIK and RIP1.

6.17 Summary

This thesis has explored the function of Pellino1 in human airway epithelial cells and specifically in terms of its regulation in response to pro-inflammatory stimuli, the phenotype of Pellino1 knockdown cells and its mechanism of action. This is the first description of the roles of Pellino1 in primary human cells and in response to an important human pathogen that is involved in the exacerbation of one of the most common respiratory diseases, asthma.

I hypothesised that Pellino1 and the rest of the Pellino family would be regulated in response to pro-inflammatory stimuli and as the tools for the measurement of Pellino proteins are extremely limited, regulation was measured at the mRNA level. Pellino1 showed evidence of modest induction after stimulation with selected pro-inflammatory stimuli in peripheral blood neutrophils and primary bronchial epithelial cells and this induction is not seen in the human airway epithelial cell-line BEAS-2B cells. Regulation of Pellino2 and Pellino3a and Pellino3b were measured using RT-PCR, which will only measure dramatic changes in transcript levels and it was found that other members of the Pellino family did not exhibit dramatic changes in transcript levels in response to pro-inflammatory stimuli in either epithelial cells or peripheral blood neutrophils.

Pellino1 stable knockdown in BEAS-2B cells was unsuccessful as knockdown was variable and this may have been due to an essential role of Pellino1 in the growth and development of these cells as stable MyD88 clones created using the same technique exhibited consistent target gene knockdown.

In support of my hypothesis, transient knockdown of Pellino1 showed that Pellino1 is an important mediator of the TLR/IL-1R signalling pathway in human. Specifically in BEAS-2B cells, Pellino1 played a role in both IL-1 and TLR3 signalling, however in PBECs Pellino1 seems to function independently of IL-1 but plays a role specifically in the TLR3 pathway. Data from PBECs support that from the Pellino1 knockout mouse, which shows a significant reduction in IL-6 and TNF production in response to poly(I:C) and LPS; however Pellino1 is dispensable for IL-1 signalling and IFNβ production. Data from PBECs supported my final hypothesis that Pellino1 is involved responses to viral pathogens, however Pellino1 knockdown in BEAS-2B cells infected with RV1B had no effect on cytokine production.

Differences between the phenotypes in BEAS-2B cells and PBECs could possibly be due to the differences in signalling pathways that are activated by TLR3 in each cell type. BEAS-2B

cells were shown to activate canonical NF-κB signalling in response to poly(I:C), however this was absent in PBECs and these cells may utilise the non-canonical NF-κB signalling pathway. The Pellino1 target is still yet to be elucidated as RIP1 seemed to play a surprising negative regulatory role in the TLR3 pathway in PBECs.

Data from this thesis suggest that Pellino1 could act as a potential drug target for the treatment of airway diseases such as asthma. This was supported by Pellino1 knockdown in PBECs isolated from asthma patients showing a reduction in IL-8 and a preservation in response to poly(I:C) stimulation allowing for down-regulation of neutrophilic inflammation whilst retaining antiviral immunity.

Appendix 1

1. PCR and molecular cloning

50 X TAE Buffer

	Final Concentration	Mass/Volume
Tris Base	1.67 M	242 g
EDTA	50 nM	37.2 g
Acetic Acid		57.1 ml
Water		To 1 litre

Stored at room temperature and diluted to 1 X with water.

2. TOPO TA Cloning

Reagent	Composition	Storage
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	-20°C
Topo Vector	10 ng/µl plasmid DNA in: 50% glycerol	-20°C
	50 mM Tris-HCl, pH 7.4 (at 25°C)	
	1 mM EDTA	
	1 mM DTT	
	0.1% Triton X-100	
	100 µg/ml BSA	
	phenol red	
S.O.C Medium	2% Tryptone	+4°C
	0.5% Yeast Extract	
	10 mM NaCl	
	2.5 mM KCl	
	10 mM $MgCl_2$	
	10 mM $MgSO_4$	
	20 mM glucose	
X-gal	40 mg/ml in:	+4°C
	Dimethylformamide (DMF)	

Reagent	Composition	Storage
LB Broth (pH 7.5)	1 % Tryptone 0.5 % Yeast Extract 85 mM NaCl 0.1 % Glucose	Autoclaved and stored at +4°C
LB Agar (pH 7.5)	1 % Tryptone 0.5 % Yeast Extract 0.5 % Yeast Extract 85 mM NaCl 0.1 % Glucose 1.5 % Agar	Autoclaved and stored at +4°C

3. Western Blotting

Phosphatase Lysis Buffer

Reagent	Concentration	Mass/Volume
Tris Base pH 7.5	50 mM	0.3 g
Sodium Fluoride	50 mM	0.1 g
B-glycerophosphate	50 mM	0.54 g
Sodium Orthovanadate	10 mM	0.09 g
Triton X-100	1 %	500 µl
Water		Up to 50 ml

Stored at 4°C. Add 1 mM PMSF (stock 100 mM in isopropanol) and 1:100 protease inhibitors (Calbiochem) before use.

SDS PAGE Lysis Buffer

Reagent	Concentration	Mass/Volume
Tris-HCl pH 6.8	100 mM	1 ml of 1M
Dithiothreitol (DTT)	200 mM	2 ml of 1 M
SDS	4 %	4 ml of 20 %
Glycerol	20 %	2 ml of 100 %
Bromophenol Blue	0.2 %	1 ml of 2 %
Water		to 10 ml

Stored at -20°C

SDS PAGE Gels (makes 2 gels)

Resolving Gel	**15 %** (<20 kDa)	**12%** (15-50 kDa)	**10 %** (40-120 kDa)
Distilled water	3.4 ml	4.9 ml	5.9 ml
30 % Acrylamide	7.5 ml	6 ml	5 ml
1.5 M Tris (pH 8.8)	3.8 ml	3.8 ml	3.8 ml
20 % SDS	75 µl	75 µl	75 µl
20 % APS	150 µl	150 µl	150 µl
TEMED	6 µl	6 µl	6 µl

Stacking Gel (5 %)	
Distilled water	2.8 ml
30 % Acrylamide	830 µl
1 M Tris pH 6.8	1260 µl
20 % SDS	25 µl
20 % APS	50 µl
TEMED	5 µl

10 X SDS Running Buffer

Reagent	Concentration	Mass/Volume
Tris	0.2 M	30.3 g
Glycine	1.92 M	144 g
SDS	20 %	10 g
Water		To 1 litre

Stored at room temperature

10 X Transfer Buffer

Reagent	Concentration	Mass/Volume
Tris	0.25 M	36.3 g
Glycine	2.4 M	181.25 g
Water		To 1 litre

Stored at room temperature. To make 1 litre of 1 X Transfer buffer, take 80 ml of 10 X and mix with 200 ml methanol and 720 ml water.

PBS-tween

Reagent	Concentration	Mass/Volume
Phosphate buffered saline (PBS)		1 litre
Tween®-20	0.2 %	2 ml

Stored at room temperature

Blocking Buffer

Reagent	Concentration	Mass/Volume
Powdered milk	5 %	2.5 g
PBS		To 50 ml

Stored at +4°C for up to 24 hours

Antibody Buffer

Reagent	Concentration	Mass/Volume
Powdered milk	5 %	2.5 g
PBS-tween		To 50 ml

Stored at +4°C for up to 24 hours

4. Quantitative PCR primer-probe sequences

Target	Type	Sequence
IFNβ	Fwd	5'-CGCCGCATTGACCATCTA-3'
	Rvs	5'-TTAGCCAGGAGGTTCTCAACAATAGTCTCA-3'
	Probe	5'-TCAGACAAGATTCATCTAGCACTGGCTGGA-3'
IFNλ1	Fwd	5'-GGACGCCTTGGAAGAGTCACT-3'
	Rvs	5'-AGAAGCCTCAGGTCCCAATTC-3'
	Probe	5'-AGTTGCAGCTCTCCTGTCTTCCCCG-3'
IFNλ2/3	Fwd	5'-CTGCCACATAGCCCAGTTCA-3'
	Rvs	5'-AGAAGCGACTCTTCTAAGGCATCTT-3'
	Probe	5'-TCTCCACAGGAGCTGCAGGCCTTTA-3'
RV1B	Fwd	5'-GTGAAGAGCCSCRTGTGCT-3'
	Rvs	5'-GCTSCAGGGTTAAGGTTAGCC-3'
	Probe	5'-TGAGTCCTCCGGCCCCTGAATG-3'

Primer-probe sequences used for qPCR

Target specific primer-probe sets for use in qPCR were ordered from Sigma-Aldrich and probes contain the FAM fluorescent dye and the TAMRA quencher. The table shows the sequences for the forward (Fwd), reverse (Rvs) and probes used for qPCR reactions.

5. ELISA Buffers

Coating Buffer (pH 7.2-7.4)

Reagent	Concentration	Mass/Volume
NaCl	0.14 M	8.18 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	8.1 mM	1.15 g
KH_2PO_4	1.5 mM	0.2 g
Water		To 1 litre

Stored at +4°C

Wash Buffer (pH 7.2)

Reagent	Concentration	Mass/Volume
NaCl	0.5 M	292.2 g
NaH_2PO_4	2.5 mM	3 g
Na_2HPO_4	7.5 mM	10.7 g
Tween®-20	0.1 %	10 ml
Water		To 10 litres

Stored at +4°C

6. Spearman Karber formula

The Spearman Karber formula was used to determine the $TCID_{50}$ of the RV1B viral batch used in functional experiments. The Spearman Karber formula is **M= xk +d[0.5-(1/n)(r)]** where xk is the dose of the highest dilution, d is the spacing between dilutions, n is the number of well of each dilution and r is the sum of the negative responses.

7. Manufacturing lentivirus using the BLOCK-iT™ Inducible H1 Lentiviral RNAi System

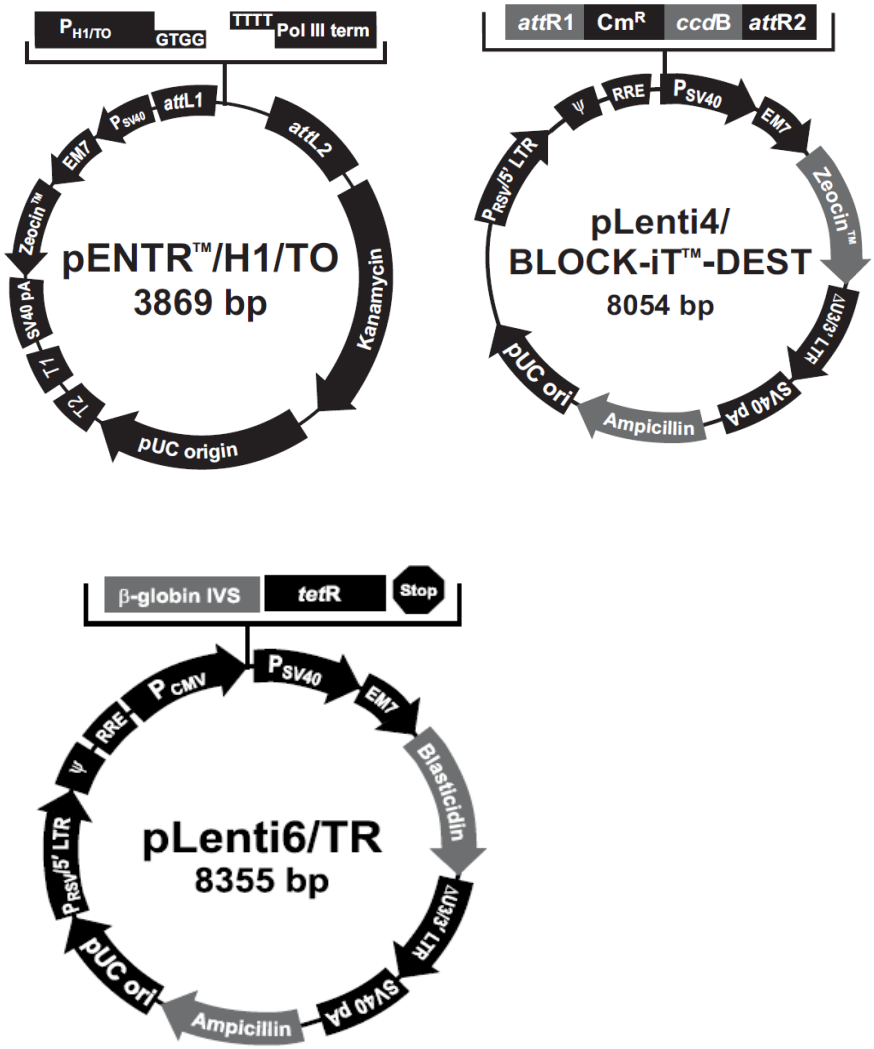
Manufacturing of the entry clone pENTR™/H1/TO

Reagent	Composition
pENTR™/H1/TO vector, linearised	0.75 ng/µl plasmid DNA in: 10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0
10X Oligo Annealing Buffer	100 mM Tris-HCl, pH 8.0 10 mM EDTA, pH 8.0 1 M NaCl
5X Ligation Buffer	250 mM Tris-HCl, pH 7.6 50 mM $MgCl_2$ 5 mM ATP 5 mM DTT 25% (w/v) polyethylene glycol-8000
T4 DNA ligase	1 (Weiss) U/µl in 10 mM Tris-HCl, pH 7.5 50 mM KCl 1 mM DTT 50% (v/v) glycerol

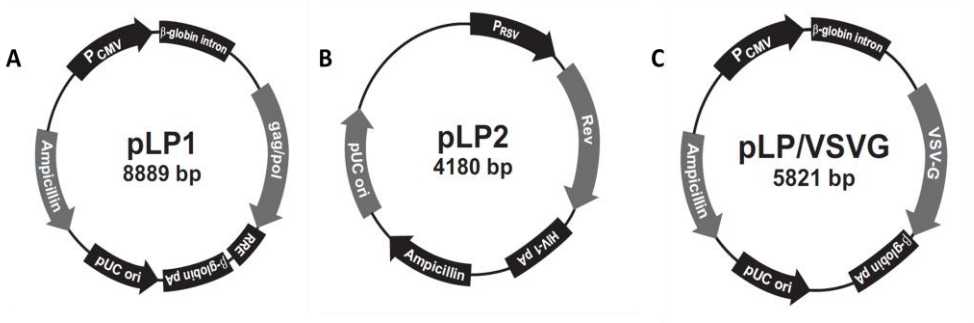
Reagents for manufacturing of the entry clone pENTR™/H1/TO

The table outlines the composition of the reagents supplied in the BLOCK-iT™ Inducible H1 Lentiviral RNAi System used in the manufacture of the double-stranded oligo encoding a shRNA that is used to target specific genes for silencing via the RNAi pathway and its subsequent cloning into the pENTR™/H1/TO plasmid.

8. **pENTR™/H1/TO entry vector, pLenti4/BLOCK-iT™-DEST vector and pLenti6/TR plasmid plasmid maps**



9. **ViraPower™ Packaging Mix plasmid maps**



10. Features of pENTR™/H1/TO entry vector

Feature	Benefit
*rrn*B T1 and T2 transcription terminators	Reduces potential toxicity in *E. coli* by preventing basal expression of the double-stranded oligonucleotide of interest.
SV40 polyadenylation site	Allows transcription termination and polyadenylation of mRNA
Zeocin™ resistance gene	Allows stable selection in mammalian cells and prokaryotes.
EM7 promoter	Synthetic prokaryotic promoter for expression of the Zeocin™ resistance marker in *E. coli*
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen
M13 forward (-20) priming site	Allows sequencing of the plasmid
*att*L1 and *att*L1 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the H1/TO RNAi entry construct with the pLenti4/BLOCK-iT™ destination vector
H1 forward priming site	Allows sequencing of the plasmid
Human H1/TO promoter	Hybrid promoter consisting of the human H1 promoter and two tetracycline operator ($tetO_2$) sequences for RNA Polymerase III-dependent regulated expression of the short hairpin RNA.
5' overhangs	Allows ligase-mediated directional cloning of the double-stranded oligonucleotide of interest
Pol III terminator	Allows efficient termination of RNA polymerase III-dependent transcription
M13 reverse priming site	Allows sequencing of the plasmid
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*
pUC origin of replication	Permits high-copy replication and maintenance in *E. coli*

11. Features of the pLenti4/BLOCK-iT™-DEST vector

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral production of viral mRNA
HIV-1 truncated 5' LTR	Permits viral packaging and reverse transcription of the viral mRNA
5' splice donor and 3 acceptors	Enhances the biosafety of the vector by facilitating removal of the ψ packaging sequence and RRE (see below) such that the expression of the gene of interest in the transduced host cell is no longer Rev-dependent.
HIV-1 psi (ψ) packaging signal	Allows viral packaging
HIV-1 Rev-dependent response element (RRE)	Permits Rev-dependent nuclear export of unspliced viral mRNA
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the H1/TO RNAi cassette of interest from pLenti4/BLOCK-iT™ expression construct
*ccd*B gene	Permits negative selection of the plasmid
Chloramphenicol resistance Gene (Cm^R)	Allows counterselection of the plasmid
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen
EM7 promoter	Synthetic prokaryotic promoter for the expression of the selection marker in *E. coli*
Zeocin™ resistance gene	Allows selection of stably transduced mammalian cell lines
ΔU3/HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5' LTR for biosafety purposes and contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA
Bla promoter	Allows expression of the ampicillin resistance gene
Ampicillin resistance gene (β-lactamase)	Allows selection in *E. coli*
pUC origin	Permits high-copy replication and maintenance in *E. coli*

12. Features of pLenti6/TR plasmid

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/ promoter	Allows Tat-independent production of viral mRNA
HIV-1 truncated 5' LTR	Allows viral packaging and reverse transcription of the viral mRNA
5' splice donor and 3' acceptors	Enhances the biosafety of the vector by facilitating removal of the ψ packaging sequence and RRE, thus allowing the expression of the gene of interest to be Rev-independent in the transduced cell
HIV-1 ψ packaging signal	Allows viral packaging
HIV-1 Rev response element (RRE)	Allows Rev-dependent nuclear export of unspliced viral mRNA
CMV promoter	Allows high-level, constitutive expression of the Tet repressor in mammalian cells
Rabbit β-globin intron II (IVS)	Enhances the expression of the *TetR* gene in mammalian cells
TetR gene	Encodes the Tet repressor that binds to tet operator sequences to repress transcription of the gene of interest in the absence of tetracycline
SV40 early promoter and origin	Allows high level expression of the selection marker and episomal replication in cells that are expressing the SV40 large T antigen
EM7 promoter	Synthetic prokaryotic promoter for the expression of the selection marker in *E. coli*
Blasticidin resistance gene	Allows selection of stably transduced mammalian cell lines
ΔU3/HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5' LTR for biosafety purposes
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA
Bla promoter	Allows expression of the ampicillin resistance gene
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*
pUC origin	Allows high-copy replication and maintenance in *E. coli*

13. Functional benefits of ViraPower™ Packaging Mix plasmids

Feature	Plasmid	Benefit
Ampicillin resistance gene	pLP1, pLP2, pLP/VSVG	Allows selection of the plasmid in *E. coli*
pUC origin of replication	pLP1, pLP2, pLP/VSVG	Permits high copy replication and maintenance in *E. coli*
Human cytomegalovirus (CMV) promoter	pLP1, pLP/VSVG	Permits high-level expression of the HIV-1 *gag*, *pol* and VSV-G genes in mammalian cells.
Human β-globin intron	pLP1, pLP/VSVG	Enhances the expression of the *gag*, *pol* and VSV-G genes in mammalian cells.
Human β-globin polyadenylation signal	pLP1, pLP/VSVG	Allows efficient transcription termination and polyadenylation of mRNA
HIV-1 *gag* coding sequence	pLP1	Encodes the viral core proteins required for forming the structure of the lentivirus
HIV-1 *pol* coding sequence	pLP1	Encodes the viral replication enzymes required for replication and integration of the lentivirus
HIV-1 Rev response element (RRE)	pLP1	Permits Rev-dependent expression of the *pol* and *gag* genes
RSV enhancer/promoter	pLP2	Permits high-level expression of the *rev* gene
HIV-1 Rev ORF	pLP2	Encodes the Rev protein which interacts with RRE on pLP1 to induce Gag and Pol expression, and on the pLenti4/BLOCK-iT™-DEST expression vector to promote the nuclear export of the unspliced viral RNA for packaging into viral particles
HIV-1 LTR polyadenylation signal	pLP2	Allows efficient transcription termination and polyadenylation of mRNA
VSV G glycoprotein (VSV-G)	pLP/VSVG	Encodes the envelope G glycoprotein from Vesicular Stomatitis Virus to allow the production of a pseudotyped retrovirus with a broad host range

14. Sequences of the Pellino1-targeting shRNA used in the Sigma MISSION™ System

Sequences for the Pellino1-targeting shRNA packaged into each lentiviral particle and the corresponding titre expressed as transducing units per ml (TU/ml) are outlined in the table.

Virus	Sequence	Titre (TU/ml
1	CCGGCCATGTACATGGCTATCATAACTCGAGTTATGATAGCCATGTACATGGTTTTTTG	$2.4X10^7$
2	CCGGGCACCACAAGTATAGACAGTTCTCGAGAACTGTCTATACTTGTGGTGCTTTTTTG	$1.7\ X10^7$
3	CCGGGCCAAATGGAAGACATCAGATCTCGAGATCTGATGTCTTCCATTTGGCTTTTTTG	$2.0\ X10^7$
4	CCGGGCACTGTGCATATTGCTTGTACTCGAGTACAAGCAATATGCACAGTGCTTTTTTG	$2.0\ X10^7$

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