DEFINING THE PHYSIOLOGICAL AND MOLECULAR ROLE OF PELLINO3 IN TLR3 SIGNALLING

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DECLARATION

This thesis has not been previously submitted to this or any other university for examination for a higher degree. The work presented here is entirely my own except where otherwise acknowledged. This thesis may be made available for consultation within the university library. It may be copied or lent to other libraries for purposes of consultation.

Ruaidhrí Jackson

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Abstract

Innate immunity utilises a series of pathogen associated molecular pattern receptors such as the Toll-like receptors (TLRs) to detect and respond to invading microorganisms. The different TLRs play a pivotal role in the first line of defence by recognising the type of pathogenic threat encountered and by responding in kind. TLR pathogen interaction triggers the production of a number of pro-inflammatory cytokines and anti-viral mediators to initiate the process of microbial elimination. However, if left unregulated, such immunostimulatory and cytotoxic factors have the potential to cause grievous harm to the host and can facilitate and propagate autoimmune disease. Therefore, the TLR signalling response is under the control of stringent regulatory mechanisms to prevent self-harm. In this thesis, the generation and characterisation of a genetically deficient Pellino3 mouse is described. Pellino3 is a member of the highly evolutionary conserved Pellino family of E3 ubiquitin ligases. Using this model, the physiological role of Pellino3 in TLR signalling is delineated. Peli3-deficient mice display no obvious abnormalities in cytokine production in response to pathogenic bacterial and fungal ligands. Pellino3 functions as a specific regulator of anti-viral type 1 interferon production in response to double stranded viral RNA recognition by TLR3. Pellino3-deficient mice are more resistant to the pathogenic and lethal effects of encephalomycarditis virus (EMCV) infection. Pellino3 functions in a novel auto-regulatory mechanism to specifically prevent excessive TLR3 induced expression of type 1 IFNs but leaves pro-inflammatory cytokine production intact. TLR3 signalling induces Pellino3 expression which in turn interacts with TRAF6 and facilitates its polyubiquitination. This modification of TRAF6 suppresses its ability to bind and ubiquitinate a key anti-viral transcription factor IRF7, thereby down regulating the type 1 IFN response. The findings outlined in this thesis define for the first time, a physiological role for Pellino3.

Abbreviations

2'-5' OAS:	2'-5'-oligoadenylate synthetase
7-AAD:	7-Aminoactinomycin D
a/g:	protein A/protein G
A:	alanine
AP-1:	activator protein-1
APC:	llophycocyanin
APS:	ammonium persulfate
ARE:	AU rich elements
ASK1;	apoptosis signal-regulating kinase 1
ATF-3	cyclic AMP-dependent transcription factor
ATP:	adenosine triphosphate
AU:	adenylate uridylate
BAC:	bacterial artificial chromosome
BCAP:	B cell adaptor for PI3K
Bcl:	B cell lymphoma
BMDC:	bone marrow derived dendritic cells
BMDM:	bone marrow derived macrophages
BMP-4:	bone morphogenetic proteins-4
bp:	base pair
BSA:	bovine serum albumin
Btk:	Burtons tyrosine kinase
C:	carboxy; cysteine

cAMP:	cyclic adenosine 3’ 5’ -monophosphate
CD:	cluster of differentiation antigen
cDNA:	complementary DNA
CHAPS:	3-(3-Cholamidopropyl)dimethylammonio-1-propanesulfonate
CLR:	C-type lectin receptor
CM:	conditioned media
CNS:	central nervous system
CoIP:	co-immunoprecipitation
CpG:	2′-deoxyribo cytidine-phosphate-guanosine
CREB:	cAMP-responsive element binding protein
CRM-1:	chromosome region maintenance 1
DAMPs:	danger-associated molecular patterns
DC:	dendritic cell
DD:	death domain
DMEM:	Dulbecco’s modified eagle’s medium
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
dNTPs :	deoxyribonucleic triphosphates
dsRNA:	double-stranded RNA
dToll:	*Drosophila* Toll
DTT:	dithiothreitol
DUB:	deubiquitinating enzyme
E.coli:	*Escherichia coli*
E6-AP	E6-assocciated protein
EBV:	epstein bar virus

EDTA:	ethylenediaminetetraacetic acid
EIF2α	eukaryotic initiation factor 2 alpha
ELISA:	enzyme-linked immunosorbant assay
Elk1:	ETS domain-containing protein
EMCV:	encephalomyocarditis virus
EMSA:	electrophoresis mobility shift assay
ERK:	extracellular signal regulated kinase
EV:	empty vector
FADD:	fas-associated death domain
FBS:	foetal bovine serum
FHA:	forkhead-associated
FITC:	fluorescein isothiocyanate
FLTRL:	FMS-like tyrosine kinase 3 ligand
g:	gravitation
G:	guanosine
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GAS:	IFN-gamma-activated site
GM-CSF:	granulocyte-macrophage colony-stimulating factor
GOLD:	golgi dynamics domain
GSK3:	glycogen synthase kinase 3
H&E:	haematoxylin and eosin
h:	hour
H_2O_2:	hydrogen peroxide solution
H_2SO_4:	sulphuric acid
HAT:	histone acetyltransferases

HCl: hydrochloric acid

HDAC: histone deacetylases

HECT: homologous to the E6-AP carboxyl terminu

HEK: human embryonic kidney

HEPES: hydroxyeicosapentaenoic acid

HIV: human immunodeficiency virus

HPRT: hypoxanthine-guanine phosphoribosyltransferase

HRP: horseradish-peroxidase

HSP: heat shock protein

HSV: herpes simplex virus

IB: immunoblot

iBMDM: immortalised BMDM

IFN: interferon

IFNAR: type 1 IFNα/β receptor

Ig: immunoglobulin

IKK: IκB Kinase

IL: interleukin

IL-1R: IL-1 receptor

IL-1Ra: IL-1 receptor antagonist

IP: immunoprecipitation

IP-10: IFN-gamma inducible protein 10

IRAK: IL-1 receptor associated kinase

IRdye: infrared dye

IRF: interferon-regulatory factor

ISG: interferon stimulatory genes

ISGE: IFN stimulated response element

ISGF3: ISG factor 3

ISRE: interferon-stimulated response element

ITIM: immunoreceptor tyrosine-based inhibition motif

IκB: inhibitor of κB

JAK1: janus kinas 1

JNK: c-Jun N-terminal kinase

K: lysine

Kb: kilobase

KD: kilodalton

LB: lysogeny broth

LBP: LPS-binding protein

LMP1: latent membrane protein 1

LPS: lipopolysaccharide

LRR: leucine-rich repeat

LUBAC: linear ubiquitin chain assembly complex

LVP: lipo-viral particle

mA: milliamp

MALP2: macrophage activating lipopeptides 2

MAPK: mitogen activated protein kinase

MAPKK: MAPK kinase

MAPKKK: MAPKK kinase

MASP: MBL-associated serine proteases

MAVS: mitochondrial anti-viral signalling

MBL: mannose binding protein

M-CSF:	macrophage colony-stimulating factor
MD-2:	myeloid differentiation-2
MDA-5:	melanoma differentiation-associated protein 5
mDC:	myeloid/convention dendritic cells
MEF:	mouse embryonic fibroblast
MEKK:	mitogen-activated protein kinase kinase
$MgCL_2$	magnesium chloride
min:	minute
MKK:	mitogen activated protein kinase kinase
MKP1:	MAPK phosphatase
ml:	milli liter
mM:	milli molar
mRNA:	messenger RNA
MSCV:	murine stem cell virus
MsEVP:	*Melanoplus sanguinipes* entomopoxvirus
MyD88:	myeloid differentiation protein 88
Na_3VO_4:	sodium orthovanadate
NaCl:	sodium chloride
NaOH:	sodium hydroxide
NAP-1:	nucleosome assembly protein
NCOR:	nuclear receptor co-repressor
NEMO:	NF-κB essential modulator
NF-κB:	nuclear factor kappa B
ng:	nanogrammes
NK:	natural killer

NLR:	NOD-like receptor
nM:	nanomolar
NOD:	nucleotide-binding oligomerization domain
NP-40:	nonidet P-40 (octyl phenoxypolyethoxylethanol).
nt:	nucleotide
OD:	optical density
OxLDL:	oxidised low-density lipoproteins
PAGE:	polyacrylamide gel electrophoresis
Pam_2CSK4:	S-[2,3-bis(palmitoyloxy)propyl]cysteine-4
PAMP:	pathogen-associated molecular pattern
PBMC:	peripheral blood mononuclear cells
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
pDC:	plasmacytoid dendritic cell
PE:	phycoerythrin
PI3K:	phosphoinositide 3-kinase
PKR:	protein kinase RNA-activated
PMSF:	phenylmethylsulfonyl fluoride
Poly(dI-dC):	poly(deoxyinosinic-deoxycytidylic) acid
Poly(I:C):	polyinosinic-polycytidylic acid
PRD:	positive regulatory domain
PRR:	pattern-recognition receptor
R:	arginine
RA:	rheumatoid arthritis
RANTES:	regulated upon activation normal T cell expressed and secreted

RAUL:	RTA-Associated Ubiquitin Ligase
RBL:	red blood cell lysis
RBX2:	ring box protein 2
RIG-I:	retinoic acid-inducible gene 1
RING:	really interesting new gene
RIP:	receptor interacting protein
RIPA:	radioimmunoprecipitation assay
RLR:	RIG-I like receptor
RNA:	ribonucleic acid
RNAi:	RNA interference
ROS:	reactive oxygen species
RPMI:	Roswell park memorial institute
RSV:	respiratory syncytial virus
RT:	room temperature; reverse transcriptase
RTA:	replication and transcription activator
s:	serine
SARM:	sterile α and heat-armadillo motifs
SCN:	severe congenital neutropenia
SDS:	sodium dodecyl sulphate
sec:	second
SHP-1:	Src homology region 2 domain-containing phosphatase-1
siRNA:	small interfering RNA
SLE:	systemic lupus erythematosus
SMAD6:	SMA mothers against decapentaplegic 6
SOCS:	suppressor of cytokine signalling

SR-A1:	scavenger receptor-A1
ssRNA:	single-stranded RNA
STAT:	signal transducers and activators of transcription
TAB:	TAK1-binding protein
TAE:	tris-acetate-EDTA
TAG:	TRAM adaptor with GOLD domain
TAK1:	TGF-β-activated protein kinase 1
TANK:	TNFR-associated factor family member-associated NF-κB activator
Taq:	*Thermophilus aquaticus*
TAX1BP1:	tax1 binding protein
TBE:	tris-Borate-EDTA
TBK1:	TANK-binding kinase 1
TBS:	tris-buffered saline
TBST:	tris-buffered saline containing Tween 20
Tc:	threshold cycle
TE:	tris-EDTA
TEMED:	N N N' N' - Tetramethylethylene-diamine
TGF-β:	transforming growth factor-β
TIFA:	TRAF interacting protein with FHA domain
TIR:	toll-IL-1 receptor domain
TIRAP:	TIR domain containing adaptor protein
TLR:	toll-like receptor
TMB:	3 3' 5 5'-tetramethylbenzidine
TNFR:	TNF receptor
TNF-α:	tumour necrosis factor alpha

Tpl-2:	tumor progression locus 2
TRADD:	TNF receptor –associated death domain
TRAF:	TNF receptor associated factor
TRAIL:	TNF-related apoptosis-inducing ligand
TRAILR:	TNF-related apoptosis-inducing ligand receptor
TRAM:	TRIF-related adaptor molecule
TRIF:	TIR domain-containing adaptor inducing IFN-β
TRIS:	TIR-less splice variant of TIF
Tyk2:	tyrosine kinase 2
U:	uridine
ub	ubiquitin
Ubc:	ubiquitin conjugating enzyme
UBC:	ubiquitin-conjugating enzyme
UEV1a:	ubiquitin conjugating enzyme variant 1a
USP:	ubiquitin-specific protease
UV:	ultraviolet light
V:	volts
v:	volume
vPellino:	viral pellino
w:	weight
WNV:	west Nile virus
WT:	wild-type
μg:	micro grammes
μl:	micro liter
μM:	micromolar

Chapter 1:

Introduction

1.1 The Immune System

Since the initial discoveries of Robert Koch and Louis Pasteur in the late 19th century that microorganisms acted as the agents of infectious disease, the search for the mechanisms of protection to pathogens such as bacteria, viruses, fungi and protozoa have been sought. An overarching multicomponent defence organisation known as the immune system is now known to confer such host protection. The immune system is comprised of a plethora of cells, tissues and molecules that can respond to and defend the body against pathogenic infection. The initiation of the immune response is a complex, highly regulated and ordered process that tailors a selective protective reaction to specific microbial insult. The immune system is broadly categorised into two independent but fundamentally linked branches known as innate and adaptive immunity. This distinction is largely based upon the speed and longevity of the initial response, the central effector cell type functions and their specificity for individual microbial epitopes. Both are vitally important in maintaining host pathogen homeostasis as evidenced by the severe pathology witnessed in subjects with genetic abnormalities or acquired deficiencies in either component. For example, patients with inherited deficiencies in a key innate immune cell known as the neutrophil, result in a disorder known as Severe Congenital Neutropenia (SCN). SCN is characterised by severe, chronic and recurrent infections initially manifesting in one month old infants, and if left untreated, the infections often prove lethal by the second year of life (Kostmann, 1956). Conversely, T cells play a central role in the adaptive immune response and deficiencies in a subset known as $CD4^+$ helper T cells caused by Human Immunodeficiency Virus (HIV) lead to a major increase in susceptibility to life threatening infections and diseases (Hirschtick *et al.*, 1995). It is clear from above that both innate and adaptive immunity must play fundamental roles in mediating host protection. However, it is both the division of labour and interplay between these very different systems that allows efficient recognition and elimination of invading pathogens.

1.2 Innate Immunity

The innate immune system represents the first line of defence to pathogenic infection. It plays a fundamental role in the initial detection and establishment of the pro-inflammatory and anti-viral response upon pathogenic infiltration of the host. This in turn drives the activation and polarisation of adaptive immunity leading to the efficient, antigen-specific elimination of the invading microorganism and the subsequent development of adaptive immunological memory to quickly combat re-infection.

Initially, physical and chemical barriers function in tandem to prevent pathogenic microorganism establishment in the host. Mechanical blockage is achieved by the tight junctions formed between cells of the epidermis and epithelial cells lining the internal mucosal layers (Guttman and Finlay, 2009). Specialised cells in anatomically distinct regions are aided in pathogen clearance by physiological functions such as cilial action, motility, desquamation and mucus secretion which all inhibit microbial adherence to host surfaces (Basset *et al.*, 2003). Chemical defence mechanisms include the release of anti-microbial enzymes such as lysozyme which degrades bacterial peptidoglycan, a vital component of bacterial cell membranes (Dajani *et al.*, 2005). The release of growth sequestering agents such as lactoferrin, a chelating enzyme that binds iron with high affinity, also acts as an effective deterrent to microbial survival (Arnold *et al.*, 1980). In addition to such broad spectrum defence tactics, the innate immune system can also initiate immunological protection with a degree of relative specificity. The complement system is comprised of a number of soluble serum and membrane bound proteins that participate in a tightly regulated network to initiate an inflammatory cytolytic cascade in response to invading microorganisms (Dunkelberger and Song, 2010). During lectin-mediated complement activation, mannose-binding lectin (MBL) recognises and binds to carbohydrate moieties (N-acetylglucosamine, mannose, fucose or glucose) located on the surface of pathogens (Ikeda *et al.*, 1987). Upon MBL pathogen binding, MBL undergoes a conformational change allowing the recruitment of the MBL-associated

serine proteases (MASPs) which cleave key downstream complement proteins C4 and C2 facilitating complement cascade activation (Matsushita and Fujita, 1992, Ji *et al*., 1993, Thiel *et al*., 1997). This allows for the formation of the membrane attack complex which can result in damage to pathogen cellular integrity and direct cell death (Esser, 1994). In addition, complement deposition of C3b on the surface of cells from pathogens results in their opsonisation and increases their potential to be recognised and phagocytosed by the cellular component of innate immunity (Ehlenberger and Nussenzweig, 1977).

Innate immune cells comprise of phagocytic macrophages, antigen-presenting dendritic cells (DCs), tissue resident mast cells and granulocytes such neutrophils, eosinophils and basophils which all originate in the bone marrow from the myeloid progenitor hematopoietic stem cell (Akashi *et al*., 2000). Natural killer cells are also considered part of the innate immune system although unlike their compatriot leukocytes, they derive from the common lymphoid progenitor stem cell (Kondo *et al*., 1997). Although the different cells of innate immunity have their own unique and specialised functions, they all share a number of unifying traits. Cells of innate immunity are immediately ready to respond to pathogenic insult, do not form immunological memory and do not require selection and expansion before mediating their immunological function. Unlike the T and B cells of adaptive immunity, innate leukocytes cannot initiate a specific immune response against every possible foreign antigen present on the pathogen. This is because the functional genes encoding key subunit chains of T and B cell receptors are assembled by somatic recombination from a multitude of separate gene segments (Siu *et al*., 1984, Brack *et al*., 1978). This can therefore give rise to a potentially unlimited number of different specificity T and B cells receptors for virtually any pathogenic antigen. Upon recognition of a pathogen antigen, displayed on major histocompatibility complex (MHC) class receptors found on innate immune leukocytes, the adaptive immune cell undergoes selection and clonal expansion to allow efficient combat and elimination of cells displaying the pathogenic antigen. (Zinkernagel and Doherty, 1974, Langhoff and Steinman, 1989). Although this system is extremely efficient and specific for

pathogen clearance, alone it would be ineffective to maintain a healthy host. A mature adaptive immune response can take days to develop and requires not only direct pathogenic antigen presentation by innate immune cells, but it is also completely reliant on innate immune derived cytokines and co-stimulatory molecules to direct an appropriate adaptive immune response. In contrast, cells of the innate immune response are fully capable of rapid induction of the immune responses whereby they can directly attack the invading microorganism and begin the process of adaptive immune activation. This near instant response is facilitated by their ‘innate’ ability to detect and distinguish between different types of pathogen and orchestrate the appropriate immunological response to that given infection.

1.3 Pattern Recognition Receptors (PRRs) and Pathogen Associated Molecular Patterns (PAMPs)

Central to innate immune cell functionality is a series of germline encoded pattern-recognition receptor (PRRs) families (Janeway and Medzhitov, 2002). The PRRs can detect and differentiate between a vast array of microorganisms by sensing evolutionary conserved structures that are vital for pathogen viability but not found in host physiology and have been termed pathogen associated molecular patterns (PAMPs) (Janeway, 1989). Bacterial PAMPs are often displayed on their cell surface allowing innate immune PPRs to recognise them extracellularly. They include peptidoglycan which is found on nearly all bacterial surfaces, a key component of the gram negative bacterial cell wall lipopolysaccharide (LPS) and flagellin, the major constituent protein of the motile bacterial flagella (Schwandner *et al*., 1999, Poltorak *et al*., 1998, Hayashi *et al*., 2001). Like bacteria, fungal pathogens also have the ability to trigger a PRR response due to the many PAMPs displayed on their cellular exteriors. Their cell walls are commonly constructed of chitin, glucan, mannan and mannoproteins and all have the ability to trigger an innate immune response (Reese *et al*., 2007, Kataoka *et al*., 2002, Tada *et al*., 2002, Bozza *et al*., 2009). In contrast, viral PAMPs are often only evident during their replication cycle

which is predominantly inside the host cell. In accordance, PRRs for viral PAMPs are often located inside the host cytoplasm or on endosomal membranes. Depending on the viral infection, these PAMPs include single stranded (ss) and double stranded (ds) viral RNA and DNA, which on their own or acting in unison with other viral characteristics (such as unmethylated DNA CpG motifs or tri-phosphate caps on dsRNA), alert innate immunity to infection (Lund *et al*., 2004, Stetson and Medzhitov, 2006, Hemmi *et al*., 2000, Hornung *et al*., 2006, Alexopoulou *et al*., 2001). It is clear from the above that a multitude of very diverse PAMPs exist to alert the immune system to infectious microorganisms. Therefore it is not surprising that a multitude of PRRs have also co-evolved to detect and direct a relatively specific innate immune response to that infection. To date, 4 families of PRRs have been characterised. They include the transmembrane Toll-like Receptors (TLRs), C-type Lectin Receptors (CLRs), cytosolic Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerisation domain receptors (NOD)-like receptors (NLRs). Differential PRR activation can lead to diverse signalling cascades culminating in the activation of distinct transcription factors and the alteration of gene expression. PRR signalling can trigger the upregulation of genes encoding pro-inflammatory cytokines, type 1 interferons (IFNs), chemokines, antimicrobial proteins, costimulatory molecules and proteins involved in the modulation and regulation of PRR signalling. Together, these mediators initiate and control the innate immune response and can create an inflammatory microenvironment to direct and dictate the adaptive immune response. Therefore, the initial correct recognition of the invading pathogen is absolutely essential for overall host protection.

1.4 Toll-like Receptors (TLRs)

The identification of the mammalian PRR system began with the discovery of Toll, a transmembrane receptor expressed in the insect *Drosophila melanogaster* that functions in dorsal-ventral embryonic polarity (Hashimoto *et al*., 1988). Toll was

subsequently shown to act as the key receptor for the insect immune response to fungal pathogens. It was required for the upregulation of anti-microbial mediators through a signalling pathway that was homologous to mammalian cytokine activation by Nuclear Factor-kappa B (NF-κB) (Lemaitre *et al.*, 1996). The first mammalian Toll homologue cloned (originally annotated human Toll (hToll) now known as TLR4) showed that like its ancestrally linked insect protein, it too functioned in the induction of an anti-microbial response (Medzhitov *et al.*, 1997a). Further characterisation showed that the mammalian TLR4 was a *bona fide* PRR and functioned by detecting the bacterial PAMP LPS to initiate the innate immune response (Hoshino *et al.*, 1999, Poltorak *et al.*, 1998, Shimazu *et al.*, 1999). Taken together these data provided experimental evidence for the theory that PRRs detect PAMPs to initiate the innate immune response as first hypothesised by Charles Janeway Jr. in 1989 (Janeway, 1989). Such studies lead to an intense period of research into TLRs and the elucidation of their complex signalling pathways.

To date, using sequence homology, genetic and biochemical approaches, 10 human and 12 murine functional TLR genes have been identified. TLR1-9 are conserved and functional in both species. TLR10 has lost functionality in the mouse due to an inactivating retroviral insertion while TLR11-13 are absent in the human genome (Kawai and Akira, 2009). TLRs are type 1 transmembrane receptor glycoproteins. Their location within the cell is dependent on the PAMPs in which they are designed to detect. TLRs responsible for recognition of extracellular pathogens are located on the cell surface, while others remain sequestered on intracellular endosomes to mediate viral replication surveillance. Consequently, the TLR N-terminus is located on the exterior of the cell or inside the lumen of an intracellular compartment. This TLR ectodomain is responsible for PAMP recognition and ligand binding and specificity. The presence of a varying number (19-25) of tandem Leucine Rich Repeat (LRR) motifs, each of which is 24–29 amino acids in length mediates specific ligand binding by the different TLRs (Bell *et al.*, 2003). Upon TLR-LRR recognition of its associated ligand(s) (see table 1.1), TLR hetero/homo-dimerisation, or in the case of weakly pre-associated complexes, a conformational change occurs

triggering the association and activation of the cytoplasmic signalling domain. This domain is homologous to that of the interleukin 1 receptor (IL-1R) and is termed the Toll/IL-1R homology (TIR) domain (Slack *et al*., 2000, Medzhitov *et al*., 1998b). The close association of the TLRs TIR domains allows for the recruitment of key signalling TIR adaptor molecules, myeloid differentiation primary response protein 88 (Myd88), TIR doman containing adaptor protein (TIRAP), TIR domain contain adaptor protein inducing interferon-β (IFN-β) (TRIF) and TRIF related adaptor molecule (TRAM) (Feng *et al*., 2003, Fitzgerald *et al*., 2001, Medzhitov *et al*., 1998b, Fitzgerald *et al*., 2003b, Yamamoto *et al*., 2002c). The TIR adaptor proteins play a critical and non-redundant role in TLR signalling and it has recently been proposed that differential recruitment and activation of the TIR adaptors by different TLRs plays a fundamental role in the specificity of the innate immune response (Akira *et al*., 2006). The TLRs family can therefore be subdivided into groups based on their ligand specificity (West *et al*., 2006).

Receptor	Ligands	Origin of ligands
TLR1	Tri-acyl lipopeptides (Pam_3CSK4)	Bacteria, mycobacteria
	Soluble factors	Neisseria meningitides
TLR2	Lipoprotein/lipopeptides	A variety of pathogens
	Di-acyl lipopeptides (Pam_2CSK4)	Mycoplasma
	Tri-acyl lipopeptides (Pam_3CSK4)	Bacteria, mycobacteria
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	A phenol-soluble modulin	*Staphylococcus epidermidis*
	Glycoinositolphospholipids	*Trypanosoma cruzi*
	Glycolipids	*Treponema maltophilum*
	Porins	Neisseria
	Zymosan	Fungi
	Atypical LPS	*Leptospira interrogans*
	Atypical LPS	*Porphyromonas gingivalis*
	LPS	*Porphyromonas gingivalis*
	HSP70	Host
TLR3	Double-stranded RNA	Virus
	Polyinosinic-polycytidylic acid (Poly(I:C))	Synthetic dsRNA
	Polyadenylic–polyuridylic acid (Poly(I:U))	Synthetic dsRNA
TLR4	LPS	Gram-negative bacteria
	Taxol	Plant
	Fusion protein	RSV
	Envelope proteins	MMTV
	HSP60	*Chlamydia pneumoniae*
	HSP60	Host
	HSP70	Host
	Type III repeat extra domain A of fibronectin	Host
	Oligosaccharides of hyaluronic acid	Host
	Polysaccharide fragments of heparan sulfate	Host
	Fibrinogen	Host
	Fetuin-A	Host
TLR5	Flagellin	Bacteria
TLR6	Di-acyl lipopeptides (Pam_2CSK4)	Mycoplasma
TLR7	Imidazoquinoline	Synthetic compounds
	Loxoribine	Synthetic compounds
	Bropirimine	Synthetic compounds
	Single-stranded RNA	Virus
TLR8	Imidazoquinoline	Synthetic compounds
	Single-stranded RNA	Virus
TLR9	CpG DNA	Viruses, Bacteria and Protozoa

Table 1.1 Toll-like receptors and their ligands.

Adapted from Akira and Takeda, 2004.

1.4.1 TLR1/2/6 Heterodimers Recognise a Broad Spectrum of Microbial Products

Unlike the other members of the TLR family, TLR1/2/6 are unique in that they do not preferentially form homodimers. Instead, TLR1/2 and TLR2/6 form heterodimers allowing the recognition of a diverse array of microbial PAMPs at the cell surface (Ozinsky et al., 2000a, Wyllie et al., 2000). Interestingly, the genetic makeup of TLR1/2/6 shares a high degree of sequence homology (~66%) and a similar genomic structure. Furthermore, the genes are all located in tandem on the same chromosome suggesting the 3 individual genes evolved and diverged from a single common ancestor to allow these TLRs to attain a greater diversity in ligand specificity (Farhat *et al*., 2008, Takeda *et al*., 2003, Du *et al*., 2000). Therefore, it is not surprising that TLR2 has the greatest repertoire of ligands of all the TLRs and has been implicated in the direct molecular discrimination of different pathogens (Underhill *et al*., 1999).

Although TLR2 was originally thought to confer host recognition to LPS, it was found contaminating lipoproteins in the LPS preparations were the actual detected ligands (Hirschfeld *et al*., 2000, Yang *et al*., 1998). In addition TLR2-deficient mice were shown to be highly susceptible to infection by the Gram positive bacteria *Staphylococcus aureus* (Takeuchi *et al*., 2000b). TLR2 recognizes lipopeptides that are anchored to the bacterial membrane by lipid chains covalently attached to the N-terminal cysteine (Manavalan *et al*., 2011). It has recently been shown that lipoproteins from Gram positive and negative bacteria differ in their specificity for the TLR2 heterodimer. Lipopeptides from Gram-negative bacteria have three lipid chains, while lipoproteins from Gram-positive bacteria and mycoplasm have only two such chains. Two of these chains are attached to the lipopeptide glycerol through an ester bond, which is in turn connected to the sulfur atom of the N-terminal cysteine and is said to be di-acylated. The third lipid chain of the Gram-negative bacteria is attached to the N-terminal of the lipopeptides via an amide bond causing the cysteine to become tri-acylated, a modification absent in Gram-positive bacteria.

(Muhlradt *et al*., 1997, Shibata *et al*., 2000). Such distinctions in the lipoprotein composition allowed for the generation of specific synthetic analogues for TLR1/2 and TLR2/6 activation. The synthetic di-acylated cysteine group analogue Pam_2CSK_4 and the tri-acylated cysteine group analogue Pam_3CSK_4 have been shown to specifically induce TLR2/6 and TLR1/2 heterodimers respectively, both in functional proinflammatory cytokine production and at the crystal structure level (Jin *et al*., 2007, Kang *et al*., 2009, Takeuchi *et al*., 2002, Takeuchi *et al*., 2001). This strict paradigm of TLR2/6 recognition of di-acyalted bacterial components has been somewhat complicated with the discovery of the role of CD36 in TLR signalling. CD36 acts an accessory receptor for R-enantiomer of macrophage-activating lipopeptide 2 (MALP2) from *Mycoplasma pneumonia*, a di-acylated lipoprotein, but has no effect on TLR2/6 recognition of Pam_2CSK_4 highlighting the complexity and specificity of ligand recognition exhibited during TLR2 signalling. (Hoebe *et al*., 2005).

TLR2 also facilitates recognition of the mycobacterial glycolipid lipoarabinomannan, the phenol-soluble modulin of *Staphylococcus epidermidis*, the Meningococcal porin PorB from Neisseria meningitidis and lipoteichoic acids from S*taphylococcus aureus* and *Bacillus subtilis* (Means *et al*., 1999, Hajjar *et al*., 2001, Opitz *et al*., 2001, Massari *et al*., 2006).

In addition to bacterial recognition, TLR2 also participates in the detection of fungal pathogens. TLR2/6 heterodimers are also required for the recognition of the cell wall component zymosan of *Saccharomyces cerevisiae* (Ozinsky et al., 2000b). Interestingly, TLR2 participates with the C-type lectin receptor Dectin-1 to modulate independent but synergistic signalling in response to zymosan. While Dectin-1 induces reactive oxygen species and phagocytosis, TLR2/6 and Dectin-1 cooperate in NF-κB activation of pro-inflammatory cytokines (Gantner *et al*., 2003). In addition, the ability of TLR2 to cooperate with not just TLR1/6 but other families of PRRs suggest that the vast array of ligands recognised and distinguished by TLR2 may only be one facet of its function. It may play a role in the fine tuning of the

innate immune response during the complex signalling cascades exhibited during pathogenic infection.

1.4.2 TLR3 Detects Viral dsRNA

In contrast to the heterogeneity of ligands that the TLR2 complex recognises, TLR3 is highly specific for dsRNA. TLR3 ligand binding is dependent on the formation of a TLR3 homodimerisation complex in which the receptors themselves interact weakly but share a high affinity interaction with their dsRNA ligand (Wang *et al*., 2010). Interestingly, more than one TLR3 dimer can bind a single dsRNA molecule allowing the number of participating receptors and signalling complexes to increase as dsRNA strand length increases (Leonard *et al*., 2008). Upon TLR3 dimersiation the formation of a specialised TIR domain containing platform is created. Unlike the other TLRs, TLR3 does not require Myd88 to initiate downstream signalling and instead solely relies on a TRIF-mediated signalling cascade. (Yamamoto *et al*., 2003a, Hoebe *et al*., 2003). TRIF-dependent signalling results in the production of both pro-inflammatory and anti-viral mediators in order to combat the viral infection detected.

The discovery that dsRNA was detected by TLR3 was inferred from TLR3-deficient animal studies in which the TLR3 knockout animals displayed reduced cytokine production and were resistant to experimentally lethal dsRNA administration (Alexopoulou *et al*., 2001). Furthermore, evidence for TLR3 detection of naturally occurring viral RNA was also appreciated. The rotavirus has a dsRNA genome and is responsible for common infectious gastroenteritis in children. It has been shown that the intestinal epithelium of neonatal mice has a low expression of TLR3 and this is attributed to increases in susceptibility to rotaviral infection. TLR3 levels increase dramatically after weaning and are associated with increased protection from the disease. Importantly, mice deficient in TLR3 do not show this decrease in rotavirus susceptibility indicating the protective role of TLR3 in sensing dsRNA viruses (Pott *et al*., 2012). Although only 8 families of viruses have dsRNA genomes, TLR3 is not limited to their detection alone (Mertens, 2004). dsRNA is often a key intermediate

in viral replication of positive strand ssRNA virus, such as the encephalomyocarditis virus (EMCV) and DNA viruses, such as the herpes simplex virus 1 (HSV) (Weber *et al*., 2006). Interestingly, TLR3 does not detect or respond to dsRNA indiscriminately. Host dsRNA is highly modified with a large number of methyl-modified nucleosides and such methylated dsRNA exhibit an abrogated TLR response (Karikó *et al*., 2005). Furthermore, polyinosinic:polycytidylic acid (Poly(I:C)) is a synthetic analogue of dsRNA routinely used to activated TLR3 experimentally. The unmodified nature of the Poly(I:C) is highly recognisable by TLR3 which preferentially detects this structure and differs largely from those recognised by the other dsRNA-binding proteins (Okahira *et al*., 2005). Although a number of other dsRNA detection mechanisms exist, such as the RIG-I and MDA-5 pathways, the role of TLR3 in sensing extracellular and cell associated dsRNA on necrotic and virally infected cells is paramount (Schulz *et al*., 2005, McBride *et al*., 2006).

Central to TLR3 functionality is its expression profile and subcellular location. TLR3 is expressed in a wide variety of cells including professional innate immune cells such as macrophages, dendritc cells and granulocytes (excluding neutrophils that sense dsRNA by RLRs) (Alexopoulou *et al*., 2001, Tamassia *et al*., 2008) (Visintin *et al*., 2001, Yang *et al*., 2009a). In addition, TLR3 is expressed on epithelial cells, fibroblasts and endothelial cells as well as astrocytes, microgial and neuronal cells of the central nervous system (Guillot *et al*., 2005, Tissari *et al*., 2005, Matsumoto *et al*., 2002, Town *et al*., 2006, Jack *et al*., 2005, Lafon *et al*., 2006). In contrast to the other dsRNA sensors which have strict cytosolic localisation, TLR3 localisation has been shown to be somewhat promiscuous depending on the cell type and stage of viral infection. Classically, TLR3 is thought to be an intracellular receptor residing on the membrane of endosomes and this is the case for the majority of cell types such as macrophages and dendritic cells (Matsumoto *et al*., 2003). A 26 amino acid cytoplasmic linkage domain between the intracellular TIR domain and the transmembrane portion of the receptor has been discovered. This domain contains an intracellular localisation motif that restricts TLR3 to the endosome and

site directed mutagenesis targeting this region was found to result in cell surface TLR3 expression (Funami *et al*., 2004). Interestingly, although dendritic cells do not express cell surface TLR3 they are still capable of recognising exogenously added extracellular dsRNA via TLR3. Recently it has been shown that exogenous Poly(I:C) exposure to dendritic cells results in the Clathyrin-mediated endocytic internalisation of the dsRNA and its preferential sensing is by endosomal TLR3 and not that of the RLR (Itoh *et al*., 2008). In addition, endosome/phagolysosome maturation has been shown to be critical for exogenous Poly(I:C) detection as the inhibitors of acidification, bafilomycin and chloroquine, prevent TLR3 dimerisation and subsequent type 1 interferon production (de Bouteiller *et al*., 2005).

In contrast to dendritic cells, naturally expressing cell surface TLR3 has been demonstrated on fibroblasts, astrocytes and epithelial cells (Matsumoto *et al*., 2002, Jack *et al*., 2005, Sajjan *et al*., 2006). Cell surface TLR3 is also a functional antiviral sensor as pre-treatment with an monoclonal antibody against human TLR3 inhibited Poly(I:C) activation of the antiviral signalling pathway in fibroblasts (Matsumoto *et al*., 2002). Interestingly, live viral infection has the ability to not only upregulate TLR3 expression but also influence its subcellular location. Prior to respiratory syncytial virus (RSV) infection, human tracheobronchial epithelial cells have no cell surface expression of TLR3. After exposure to the virus, cell surface TLR3 is highly upregulated and extracellular membrane localisation is achieved (Groskreutz *et al*., 2006). This is thought to prime and sensitise the host to subsequent viral challenge and highlights the key role TLR3 plays in anti-viral immunity (Groskreutz *et al*., 2006).

Although great strides into the function of TLR3 in innate immune detection of viral pathogens have been made, its precise role still remains elusive. Its place in viral sensing in the context of the other dsRNA sensors remains to be fully characterised. Additionally, its role in viral detection and induction of a protective innate immune response is somewhat controversial in many circles, a problem confounded by TLR3s apparent protective and detrimental roles during different viral infection. For example, murine TLR3 plays a critical role in protection against EMCV-induced

cardiac damage and host lethality, while TLR3-induced inflammation and the resulting breakdown of the blood brain barrier plays a major role in West Nile virus (WNV) pathogenesis and encephalitis (Wang *et al.*, 2004, Hardarson *et al.*, 2007b). Despite such contrasting observations, there is no doubt that in the case of some viral infections, TLR3 is vitally important for human anti-viral defence. A dominant negative allele in TLR3 discovered in children has recently linked the PRR to protection from HSV-1-mediated encephalitis indicating TLR3 plays a non-redundant and crucial role in host protection (Zhang *et al.*, 2007). Therefore further study into TLR3s activation and regulation are vital in order to understand its core biological function in dsRNA sensing and innate immunity.

1.4.3 TLR4, the LPS Receptor and Beyond

The search for the receptor of LPS, the causative agent in endotoxin-induced septic shock, proved to be a massive undertaking culminating in the discovery of the first PRR and the awarding of the 2011 Nobel Prize to Jules Hoffmann and Bruce Beutler. The outer wall of Gram negative bacteria is an asymmetrical membrane which serves as a protective barrier from antimicrobial peptides released by the host immune system (Papo and Shai, 2005, Nikaido, 1989). Approximately 90% of the bacterial cell surface is covered in LPS and differences in the LPS structural composition confers varying susceptibility of the bacteria to different cationic anti-microbial peptides (Rosenfeld and Shai, 2006). LPS typically consists of an evolutionary conserved hydrophobic domain known as lipid A (or endotoxin), a non-repeating core oligosaccharide, and a highly variant distal polysaccharide (or O-antigen) (Rietschel *et al.*, 1994, Zahringer *et al.*, 1994). Lipid A is a phosphoglycolipid with a chemically unique architecture and represents the most highly conserved structural component of LPS and is an absolute requirement for all Gram negative bacterial cell walls (Zahringer *et al.*, 1994). It is therefore not surprising that lipid A was found to be the toxic and immunostimulatory constituent of LPS and that LPS served as the ligand for mammalian TLR4 detection (Westphal and Lüderitz, 1954, Poltorak *et al.*, 1998, Hoshino *et al.*, 1999).

Interestingly, TLR4 detection of LPS is not as simple as receptor ligand recognition. Instead a series of sequential interactions with a number of proteins is required to achieve TLR4 activation. Initially LPS is directly bound to the soluble shuttling protein, LPS-binding protein proteins (LBP), which facilitates the conversion of the oligomeric micelles of LPS to a monomer and its subsequent delivery of the ligand to a glycosylphosphatidylinositol-anchored protein known as CD14 (Wright *et al*., 1989, Wright *et al*., 1990). CD14 then associates with TLR4 and an extracellular accessory protein MD2 (Shimazu *et al*., 1999). MD2 is thought to directly bind the LPS molecule allowing TLR4 to bind the LPS/MD2 complex to trigger TLR4-mediated downstream signalling (Visintin *et al*., 2003, Nagai *et al*., 2002).

As TLR4 was the first mammalian PRR to be identified, it has been subjected to almost 15 years of intensive investigation. Interestingly, it has become clear that TLR4 not only detects a diverse array of bacterial ligands but also motifs on pathogenic viruses. Soon after TLR4 was identified as the PRR for Gram negative bacteria, reports emerged that some viral genomes encoded proteins that could negatively regulate TLR4 signalling (Bowie *et al*., 2000). These reports were closely followed with the discovery that TLR4 and CD14 could identify the fusion protein (F protein) of RSV and mount an appropriate immunological response to infection (Kurt-Jones *et al*., 2000). Furthermore, TLR4 can recognise the retroviral glycoproteins on the envelope of both the mouse mammary tumour virus and the murine leukemia virus and trigger NF-κB activation (Rassa *et al*., 2002). Recently, TLR4 was shown to be able to bind glycoproteins from viruses that cause serious pathology in humans. The Ebola virus glycoprotein present on the VP40 matrix protein of viral like particles, a structure critical for Ebola viral infection, was shown to interact with and modulate TLR4 signalling (Okumura *et al*., 2010). Lipo-viro-particles (LVP, triglyceride-rich lipoprotein like particles containing viral RNA and proteins) from Hepatits C have been also shown to interact with TLR4 and induce activation and maturation of dendritic cells leading to a viral-induced bias in the subsequent T cell subset response (Agaugue *et al*., 2007). TLR4 is therefore a key

receptor for the recognition of viral proteins and particles during the initial stage in viral infection before intracellular replication and nucleic acid exposure to the host.

An extremely important and compelling finding in PRR biology was again discovered during investigation of TLR4. The belief that PRRs could only detect molecular patterns from pathogens began to be questioned, as TLR4 was shown to recognise Heath-shock protein 60 (HSP60) of both human and microorganism origin (Ohashi *et al*., 2000, Costa *et al*., 2002). Endogenous HSP60 is usually present within the mitochondria where it acts as a chaperone protein facilitating the correct folding of oligomeric proteins (Cheng *et al*., 1989). Interestingly, during periods of cellular stress, damage or cell death, HSP60 can localise to the cell surface and it is proposed that TLR4 can recognise this danger signal and initiate innate immunity (Lehnardt *et al*., 2008).

Other endogenous ligands have also been shown to mediate TLR4 initiation of innate immunity. During tissue injury, cellular fibronectin, which contains an alternatively spliced exon encoding type III repeat extra domain A (EDA) is released from the cell and potently activates TLR4 signalling (Okamura *et al*., 2001). The role of TLR4 in autoimmunity and the onset of insulin resistance by inflammatory free fatty acids have recently been highlighted (Shi *et al*., 2006). Feutin-A a secretory liver glycoprotein has been proposed as the endogenous TLR4 ligand responsible for insulin resistance in mice on high fat diets, as knockout of either gene results in a rescue of pathology (Pal *et al*., 2012). Therefore, the first mammalian PRR/TLR discovered not only provided direct evidence of recognition of PAMPs, but has expanded our understanding of the initiation of inflammation by endogenous molecules in injury and autoimmune disease.

1.4.4 TLR5 Senses Extracellular Flagellin

TLR5 was the first PRR to be shown to bind a protein ligand. TLR5 recognises the monomeric form of flagellin, the major component of the whip-like flagellar filament responsible for bacterial locomotion (Smith *et al*., 2003, Hayashi *et al*.,

2001). Flagella are part of the sensory/motility system of bacteria and are upregulated and transported to the membrane during times of stress such as those exhibited in a nutrient depleted microenvironment or during extreme pH or saline alterations (Macnab, 2003). Flagella are critical for bacterial growth, host cellular adhesion and pathogenesis and over 50 genes under the control of a number of operons such as the master operon flhDC have been shown to be involved in flagellum production, transport and regulation (Chilcott and Hughes, 2000, Kutsukake *et al*., 1990). Such findings highlight the important role motility has on the microorganisms survival (Salazar-Gonzalez and McSorley, 2005). Therefore it is not surprising that many important human pathogenic bacteria are flagellated, including agents of gastroenteritis (*Salmonella*, *Escherichia coli* and *Campylobacter*), pneumonia (*Pseudomonas*, *Burkholderia* and *Legionella*) and invasive infections (*Proteus*, *Escherichia coli* and *Clostridium*) (Steiner, 2007).

Although Flagellin is currently the only known TLR5 ligand, due to its protein nature, it has been amenable to genetic manipulation and therefore a lot is known about the TLR5-flagellin recognition. *fliC*, the main flagellin gene in *Salmonella typhimurium*, has been subject to the most rigorous study. The flagellar filament of fliC is composed of over 20,000 subunits of a 494 amino acid flagellin and is organised into 2 domains (Ramos *et al*., 2004, McClelland *et al*., 2001). fliC has a highly conserved 140 amino-terminal and 90 carboxy terminal residues which govern polymerisation and motility and flanks a central region that is highly variable in both residue composition and size (Beatson *et al*., 2006). This conserved region is critical for flagellum motility and therefore has not undergone significant evolutionary change without deleterious effect on bacterial motility and experimental mutations in this region lead to a severe abrogation in mobility and survival (Andersen-Nissen *et al*., 2005).

This conserved region of flagellin serves as the PAMP for TLR5 recognition and pro-inflammatory cytokine production (Eaves-Pyles *et al*., 2001b, Smith *et al*., 2003). Conversely, the hypervariant region of flagellin elicits no TLR5 immunomodulation, and neither experimental mutation nor its physiological absence in *Listeria*

monocytogenes adversely affects its innate immune recognition (Hayashi *et al*., 2001, Smith *et al*., 2003). As flagellin is a protein, the exact sites of TLR5-mediated detection have been divulged using mutagenesis and structural studies. Although separated in primary sequence, the D1 domain of flagellin relates to the conserved residues of the C and N termini and forms a tertiary structure vital for TLR5-mediated detection (Yoon *et al*., 2012, Donnelly and Steiner, 2002, Murthy *et al*., 2004). Interestingly, this recognition site is not exposed on the flagellin surface when in the filamentous form and TLR5 can only detect monomeric flagellin (Smith *et al*., 2003). Although the exact mechanism for this recognition is still under investigation, it has been suggested that monomeric flagellin may be released during the hostile microenvironment of the innate immune response, during bacterial replication or during bacterial cell death (Ciacci-Woolwine *et al*., 1997, Eaves-Pyles *et al*., 2001a, Salazar-Gonzalez and McSorley, 2005). Despite the ambiguity of how TLR5 comes in contact with monomeric flagellin, it is known that TLR5 must form a homodimer to signal and the exact stoichiometry has now been uncovered (Hayashi *et al*., 2001). Structural and functional studies suggest that TLR5 forms a asymetrical inactive dimer in the absence of flagellin and upon binding of two flagellin molecules (in a 2:2 receptor:monomer ratio) TLR signalling and proinflammatory cytokine activation is achieved (Ivičak-Kocjan *et al*., 2013, Yoon *et al*., 2012, Zhou *et al*., 2012).

Although further effort is required to delineate how the precise physiological conditions occur to instigate TLR5 signalling *in vivo*, there is no doubt that TLR5 plays a fundamental role in host protection. This claim is exemplified by the recent discovery that a natural polymorphism in human ligand recognition domain of TLR5 results in an inability to respond to bacterial flagellin and results in an increased risk of serious legionnaires' disease (Hawn *et al*., 2003).

1.4.5 TLR7/8/9 the Intracellular TLRs

Intracellular TLR7/8/9 in humans and TLR7/9 in mice have evolved to detect and respond to bacterial and viral nucleic acids present inside host innate immune cells.

To a certain degree, the intracellular TLRs specificity for their natural ligands is achieved by their restricted localisation in an internal compartment from which the majority of self-nucleic acids are excluded but microorganism genetic material often frequent (Krieg and Vollmer, 2007). In addition, the transport of TLR7/9 to the endosome from the endoplasmic reticulum by UNC93B1 prevents cytosolic localisation and the requirement of endolysosome acidification for appropriated TLR7/9 responses minimise self-nucleic acid recognition in non-infected resting cells (Kim *et al*., 2008, Hacker *et al*., 1998).

Human TLR7 and TLR8 share a high level of sequence homology and are both located on the X chromosome (Akira *et al*., 2006). The first indication that TLR7/8 pathways may function in anti-viral defence came about from studies using small type 1 IFN modulating ribonucleoside analogues known as imidazoquinolines (which include imiquimod, R848 and CLO97). It was shown that TLR7 and Myd88-deficient mice exhibited a complete abrogation in imidazoquinoline-induced IFN-α and pro-inflammatory cytokine production (Hemmi *et al*., 2002). In an additional study, it was also found that human TLR8 could also respond to the imidazoquinolines R848 while murine TLR8 was non-responsive (Jurk *et al*., 2002). The natural ligands for TLR7/8 were discovered two years later to be ssRNAs in two independent reports. TLR7 was shown to be critical for the recognition of endosomal genomic ssRNA from the influenza virus by Plasmacytoid dendritic cells (pDCs) and the induction of Myd88 dependent type 1 IFN production (Diebold *et al*., 2004). In addition, they also showed that ssRNA from a non-viral origin (polyuridine) but not oligomeric RNAs could also facilitate TLR7 activation highlighting ssRNA as a natural TLR7 ligand (Diebold *et al*., 2004). In the second study, guanosine (G)- and uridine (U)-rich ssRNA oligonucleotides derived from HIV-1 was shown to stimulate dendritic cell and macrophage production of IFN-α and pro-inflammatory cytokines (Heil *et al*., 2004). Interestingly, they demonstrated that murine TLR7 and human TLR8 recognised GU-rich ssRNA specifically indicating a species specific recognition axis exists for GU-rich ssRNA detection.

Although TLR7 is fully functional in mouse models, murine TLR8 was thought to be inactive until very recently and controversies still remain about its actual role (Martinez *et al*., 2010, Bauer *et al*., 2010). Initial observations that murine TLR8 may have functionality were made when primary murine peripheral blood mononuclear cells (PBMCs) from TLR8-deficient animals were treated with the selective imidazoquinoline CLO75 in combination with polyT oligonucleotides and enhanced TLR8 activation was observed, in conjunction with a suppression of TLR7 activity (Gorden *et al*., 2006). This suggested a possible antagonist relationship between the two closely related receptors. More recently, TLR7 expression was observed to be overexpressed in TLR8-deficient dendritic cells and TLR7 signalling was hyper-reactive to various TLR7 ligands (Demaria *et al*., 2010). Furthermore, $TLR8^{-/-}$ mice displayed increased serum levels of autoantibodies against small nuclear ribonucleoproteins and ribonucleoprotein, and developed glomerulonephritis, whereas neither $TLR7^{-/-}$ nor $TLR7^{-/-}TLR8^{-/-}$ mice showed any of the phenotypic pathologies. This indicates that TLR8 has a functional role in innate immune signalling by specifically regulating aberrant TLR7-induced autoimmunity (Demaria *et al*., 2010).

Classically, TLR9 is thought to recognise unmethylated 2′-deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA islands, motifs that are highly abundant in bacterial and viral genomes but rare in mammals (Blasius and Beutler, 2010). TLR9 was originally shown to detect bacterial CpG and this was later expanded to DNA from viruses and protozoa (Hemmi *et al*., 2000, Lund *et al*., 2003, Bafica *et al*., 2006). Investigations into TLR9 biology is often conducted using two different synthetic oligonucleotides types. Type A CpG oligonucleotides are characterised by a phosphorothioate-modified poly G stretch at the 5′ and 3′ ends and a phosphodiester CpG motif in the central portion of the molecule and are potent activators of the type 1 IFN responses in pDCs (Krug *et al*., 2001, Verthelyi *et al*., 2001). In contrast, conventional phosphorothioate-modified CpG-DNAs, termed B type CpG-DNAs, do not stimulate pDCs to produce type 1 IFNs, but can activate the anti-viral cytokines in myeloid derived DC and macrophages (Schmitz *et al*., 2007b).

It is thought that the CpG A and CpG B localise to both early and late endosomes, respectively, and it is this distinction that plays a key role in the signalling pathway that is triggered (Guiducci *et al*., 2006).

Recently the dogma that TLR9 recognised the CpG motif itself was called into question, and it is now thought that TLR9 actually detects the 2′ deoxyribose phosphate backbone of the molecule and that phosphorothioate linkages nor specific sequences of the CpG motifs are required to induce a response (Haas *et al*., 2008). It is now suggested that the phosphorothioate linkages and the sequence of the CpG motifs of the synthetic ligands likely increase and influence the stability, aggregation, uptake and endosomal targeting of the DNA to mediate their variant responses (Blasius and Beutler, 2010). Finally, in addition to TLR9 recognition of pathogenic DNA, it has also been show to bind the insoluble crystal hemozoin, a by-product from the malarial heme-detoxification process that is generated after digestion of host hemoglobin by *Plasmodium falciparum* (Coban *et al*., 2010).

Consequently, the intracellular TLRs play a vital role in mediating host defence against pathogenic infection. Interestingly however, although TLR7/9 uses Myd88 to facilitate downstream signalling, the pathways employed by pDCs are very different to that employed by the TIR adaptor in TLR1/2/4/5/6 activation and therefore, in depth knowledge into these signalling cascade is vital in understanding TLR innate immune biology.

1.5 TLR Signalling Pathways

1.5.1 The TIR Adaptor Proteins

Upon recognition of their cognate PAMPs, TLRs undergo receptor dimerisation or if a low affinity pre-assembled dimer already exists, ligand binding will induce a conformational change in the receptors (Latz et al., 2007, Ozinsky et al., 2000b). Such a conformational changes facilitate the reorganisation of the cytoplasmic

domain responsible for intracellular signalling, the TIR domain (O'Neill and Bowie, 2007). The TIR domains of the TLRs undergo oligomerisation and allow the recruitment of the TIR domain containing adaptor proteins to propagate downstream intracellular signalling (Gay *et al.*, 2006). Specific and preferential adaptor recruitment by the different TLRs, their subcellular localisations and their individual affinities for interacting proteins allow the TIR adaptors to orchestrate a non-linear innate immune response to the combinations of stimuli which constitute pathogenic infection (Jenkins and Mansell, 2010).

Myd88 was originally described as a protein involved in the terminal differentiation of murine myeloid precursors cells in response to IL-6 stimulation (Lord *et al.*, 1990). Soon afterwards it became clear that a conserved sequence of cytoplasmic amino acids existed between the invertebrate Toll receptor, the mammalian IL-1R and the newly defined Myd88 protein and it was proposed to function in a conserved signal transduction pathway (Yamagata *et al.*, 1994). This proved to be the case for not only IL-1R signalling but also for TLR inflammatory responses (Medzhitov *et al.*, 1998b, Hultmark, 1994). The generation of Myd88-deficient mice proved that the adaptor protein played a fundamental physiological role in TLR4 responsiveness to LPS (Kawai *et al.*, 1999). However, although Myd88-dependent signalling is critically important for most TLR-induced NF-κB activation, a Myd88-independent signalling pathway had also been observed during TLR3- and TLR4-induced pro-inflammatory and antiviral responses (Kawai *et al.*, 2001, Alexopoulou *et al.*, 2001).

The identification of the TIR domain containing protein TRIF as a key adaptor linking TLR4 signalling to both interferon regulator factor 3 (IRF3) and NF-κB activation provided a direct mechanism to explain these Myd88-independent cytokine responses (Yamamoto *et al.*, 2002b, Oshiumi *et al.*, 2003a). TRIF was independently shown to be the only TIR adaptor that bound TLR3 after dsRNA recognition and was required for induction of interferon-β (IFN-β), a potent anti-viral cytokine (Yamamoto *et al.*, 2002b, Oshiumi *et al.*, 2003a). Furthermore, genetic disruption of the murine gene encoding TRIF provided physiological evidence for its role in TLR3 and TLR4 induction of pro-inflammatory and anti-viral

mediators in response to infection (Yamamoto *et al*., 2003a). Recently a splice variant of TRIF missing its TIR domain named TRIS has been identified. Upon TLR3 stimulation TRIF and TRIS form a heterotrimeric complex. Surprisingly, this oligomerisation is essential for both NF-κB and IRF3/7 signalling pathways and knockdown of TRIS results in reduced transcriptional activity of these transcription factors (Han *et al*., 2010). Although the exact mechanism of TRIS-dependent signalling is still unknown, this represents a unique complexity to the TRIF dependent signalling cascade not shared with the Myd88 pathway. In addition to TRIFs role in TLR3 and TLR4 signalling, it has also been implicated as a key adaptor for TLR5 signalling in intestinal epithelial cells. Flagellin stimulation allows recruitment of TRIF to TLR5 whereby in conjunction with Myd88 it mediates pro-inflammatory cytokine production (Choi *et al*., 2010). Recently, a role for TRIF in TLR1/2 recognition of *Borrelia burgdorferi*, the bacteria vector of Lymes disease, has been uncovered (Petnicki-Ocwieja *et al*., 2012). As in TLR5 signalling, TRIF dependent pro-inflammatory cytokine production is again reliant on Myd88 signalling. These findings indicate that the TIR adaptors may play cell type, infection specific and temporal roles in innate immunity beyond what is currently accepted.

In addition to Myd88 and TRIF, TLR4 also utilises two other TIR adaptors, TIRAP and TRAM. Although TIRAP was originally proposed to play a role in the Myd88 independent pathway, functional investigation into the adaptor showed it acted as a key signalling intermediate in TLR2 and TLR4 signalling by acting as a bridge between the TLR and Myd88 (Horng *et al*., 2001, Fitzgerald *et al*., 2001, Kagan and Medzhitov, 2006, Horng *et al*., 2002). Generation of TIRAP-deficient mice showed that the adaptor protein was not required for TLR3/5/7/9 or IL-1 and IL-18 signalling (Horng *et al*., 2002, Yamamoto *et al*., 2002a). To date, the role of TRAM in TLR signalling is unique as it is the only adaptor to be used by a single TLR. TRAM was initially discovered as a TIR adaptor using bioinformatical approaches (Bin *et al*., 2003). The first definitive description of TRAMs role in TLR signalling came shortly after with the observation that TRAM interacted with TLR4 and TRIF and facilitated TLR4-induced activation of IRF3 and Myd88-independent NF-κB

(Fitzgerald *et al*., 2003a). It was proposed that TRAM acted similarly to TIRAP in that it could bridge TLR4 signalling to another TIR adaptor (Oshiumi *et al*., 2003b). TRAM-deficient mice generation provided genetic evidence for its specific role in TLR4 and TRIF-dependent signal transduction (Yamamoto *et al*., 2003b). More recently, TRAM was shown to facilitate TLR4-mediated activation of anti-viral cytokine production by controlling TLR4 relocalisation from the plasma membrane (where it signals through the Myd88 dependent pathway) to an early endosome to facilitate TRIF-dependent signalling (Kagan *et al*., 2008).

Although great advances have been made in TIR adaptor-mediated signalling the roles of TIRAP and TRAM require further investigation to fully elucidate their roles in innate immune signalling. Recently, TIRAP has been shown to have independent roles to Myd88 during TLR2 and TLR4 signalling and can drive the activation of anti-inflammatory IL-10 indicating the TIR adaptors can play subtle and regulatory roles in controlling the innate immune response (Mellett *et al*., 2011). In addition, TRAM has recently been implicated as a TIR adaptor during IL-18 signalling, a receptor complex highly related to the IL-1 receptor that does not require TRAM to signal via the Myd88 dependent pathway (Ohnishi *et al*., 2012). The requirement of the additional TIR adaptor in IL-18 signalling is still unknown.

Although the elucidation of complexities of TIRAP and TRAM-mediated signalling is on-going, our knowledge of Myd88-dependent and TRIF-dependent signalling is extensive (illustrated in Fig. 1.1).

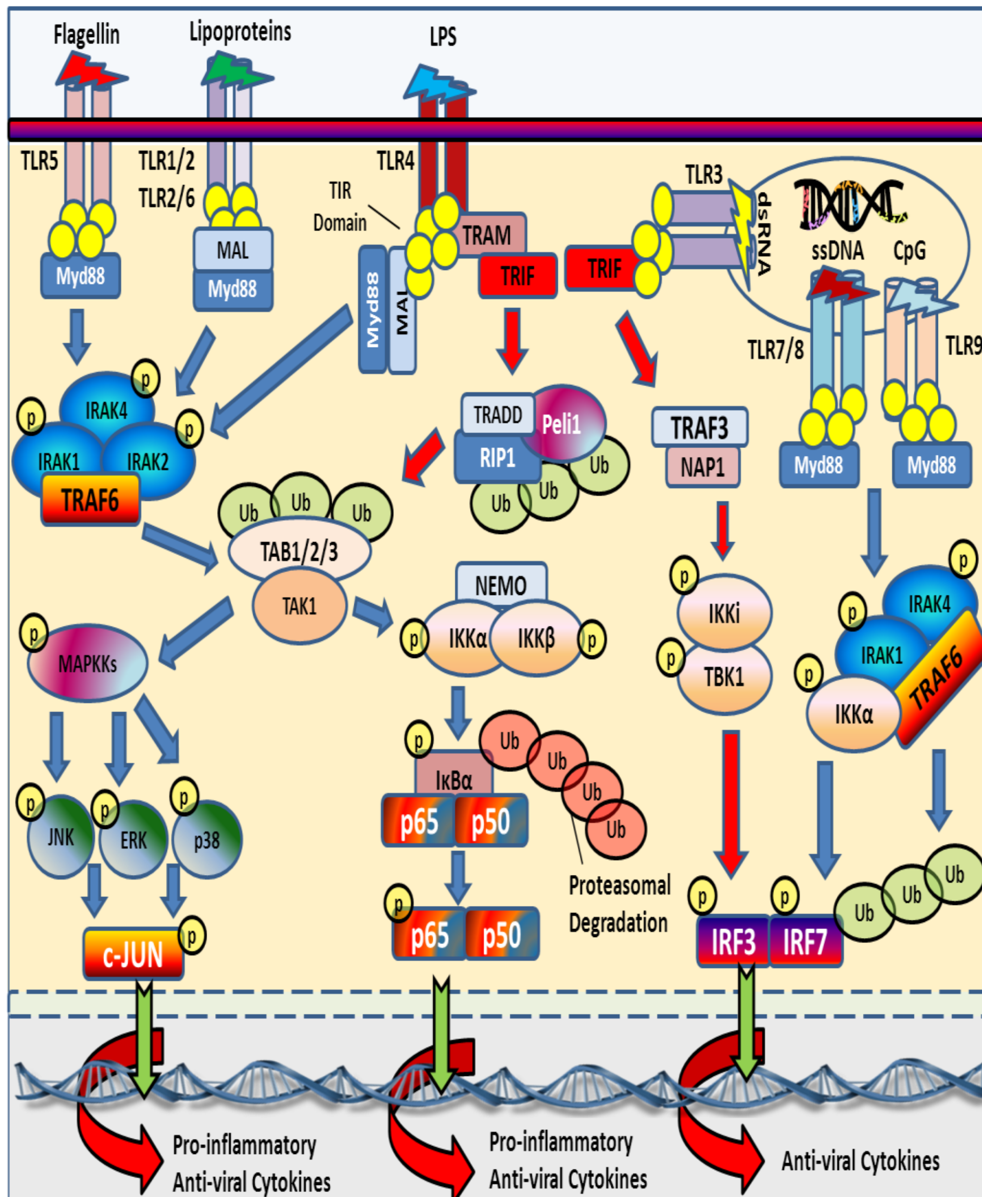


Fig. 1.1 Myd88 and TRIF-dependent TLR signalling

See section 1.5.2 for detailed Myd88 dependent signalling and section 3.1 for detailed TRIF dependent signalling pathway description. Yellow circles marked with a "p" inside represent phosphorylation events, green circles with "Ub" represents K63-linked ubiquitination while red circles with "Ub" represent K48-linked ubiquitination. Straight Blue arrows represent My88 signalling and straight red arrows represent TRIF signalling. Straight green arrows represent nuclear translocation and gene promoter binding. Curved red arrows indicate initiation of gene transcription.

1.5.2 Myd88-dependent Signalling

It is now clear that Myd88 is the critical TIR adaptor protein for all the TLRs with the exception of TLR3 (Takeuchi *et al*., 2000a, Hayashi *et al*., 2001, Heil *et al*., 2004, Hacker *et al*., 2000, Alexopoulou *et al*., 2001). Upon TLR4 dimerisation/conformational alteration, Myd88 is recruited to the proximal surface of the membrane receptor via homotypic TIR-TIR interactions with its bridging partner TIRAP (Kagan and Medzhitov, 2006). Myd88 can then in turn recruit members of the IL-1 receptor-associated kinase (IRAK) family via homophilic death domain (DD) interactions (Wesche *et al*., 1997, Burns *et al*., 1998, Medzhitov *et al*., 1998b, Muzio *et al*., 1997). Structural studies have recently uncovered the stoichiometry of these interactions. Myd88 undergoes oligomerisation into a hexameric form allowing the ordered modular recruitment of four IRAK4 molecules and four IRAK2 (or IRAK1) kinases, a complex termed the Myddosome (Suzuki *et al*., 2002, Motshwene *et al*., 2009, Lin *et al*., 2010). Formation of these complexes brings the kinase domains into close proximity for phosphorylation and activation. It is thought that IRAK4 and IRAK1 participate in the early and transient activation of NF-κB while IRAK2 is responsible for the long term sustainment of the signal (Kawagoe *et al*., 2008). While IRAK1 undergoes rapid ubiquitination and degradation upon stimulation, IRAK2 levels and activity are sustained, albeit with delayed kinetics (Yamin and Miller, 1997, Kawagoe *et al*., 2008). IRAK4 promotes the phosphorylation of IRAK1, which triggers IRAK1 auto-phosphorylation (Li *et al*., 2002, Kollewe *et al*., 2004). This triggers the phosphorylation of the E3 ubiquitin ligase Pellino1, which in turn mediates K63-linked polyubiquitination of IRAK1 enhancing its catalytic activity (Ordureau *et al*., 2008). The activated IRAK molecules are vital in linking Myd88-dependent signalling to TRAF6 activation, a key event in TLR/IL-1R functionality (Cao *et al*., 1996). TRAF6, a really interesting new gene (RING) domain containing ubiquitin ligase, acts in combination with a dimeric E2 ubiquitin conjugating enzyme known as Ubc13/Uev1A, which serves to further polyubiquitinate IRAK1 and indeed itself (Deng *et al*., 2000, Lamothe *et al*., 2007a). In addition, TRAF6 can produce unanchored free polyubiquitin chains to

activate downstream kinases to further enhance TLR-mediated signalling (Xia *et al.*, 2009). IRAK-activated TRAF6 allows the dissociation of the signalling complex from the membrane to the cytosol to facilitate further downstream signalling (Qian *et al.*, 2001). Polyubiquitylated TRAF6 can then in turn induce direct or indirect (via free chain formation) recruitment of the transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) regulatory components, TAK-1 binding protein 2 (TAB2) and TAB3 (Takaesu *et al.*, 2000, Kanayama *et al.*, 2004). This ubiquitin dependent modification allows for the activation of the kinase potential of TAK1 (Wang *et al.*, 2001). Another critical event for TLR signal transduction is the polyubiquitination of NF-κB essential modulator (NEMO) (Zhou *et al.*, 2004). A number of E3 ligases have been put forward to facilitate this modification including TRAF6 and linear ubiquitin chain assembly complex (LUBAC). However, the precise mode of NEMO ubiquitination during TLR signalling remains uncertain (Sun *et al.*, 2004, Tokunaga *et al.*, 2009, Ni *et al.*, 2008). TAK1 and NEMO are both required for the activation of the inhibitor of κB (IκB) kinases (IKKs) IKKα and IKKβ (Wang *et al.*, 2001, Mercurio *et al.*, 1997, Yamaoka *et al.*, 1998). As NEMO contains no catalytic domain and may act as a scaffold for IKK recruitment, it is TAK1 which mediates IKK phosphorylation (Wang *et al.*, 2001, Ninomiya-Tsuji *et al.*, 1999, Harhaj and Sun, 1999, Rothwarf *et al.*, 1998). Phosphorylated IKKβ in turn is recruited to the IκB proteins such as IκBα which sequester NF-κB in the cytosol (Mercurio *et al.*, 1997, Haskill *et al.*, 1991). Upon IκBα phosphorylation, it undergoes K48-linked ubiquitination by the F-box protein Slimb/β-TrCP (β-TrCP) and subsequent proteasomal-dependent degradation (Alkalay *et al.*, 1995). Liberated from its inhibitor, members of the NF-κB transcription factor family, such as p65, can in turn become phosphorylated and translocate to the nucleus to mediate direct alterations in gene expression (Sakurai *et al.*, 1999, Baeuerle and Baltimore, 1988).

Myd88 dependent signalling also has the ability to activate the mitogen and stress activated protein kinase (MAPK) pathways. In addition to IKK activation, TAK1 also functions as a MAPK kinase kinase (MAPKKK) and can phosphorylate MAPKK6 which can in turn directly activates the MAPKs c-Jun N-terminal kinases

(JNK) and p38 (Wang *et al*., 2001). TRAF6 ubiquitination also recruits a number of other MAPKKs such as MEKK3, Tpl-2 and ASK1 (apoptosis signal-regulating kinase 1) which result in the eventual phosphorylation of JNK, p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) (Banerjee *et al*., 2006, Huang *et al*., 2004, Matsuzawa *et al*., 2005). MAPK phosphorylation can lead to the direct activation of transcription factors such as is the case for JNK activation of c-JUN, or via downstream kinases as is the case for p38-MSK1/2 activation of cAMP response element binding protein (CREB) (Ananieva *et al*., 2008, Morton *et al*., 2003). In addition to transcription factor activation, MAPKs can have profound effects on the level of stability of transcripts containing adenylate/uridylate (AU)-rich elements (AREs) with p38 playing a major role in expression of mRNAs encoding pro-inflammatory cytokines (Brook *et al*., 2000, Frevel *et al*., 2003).

Myd88-dependent signalling also has the capability to activate members of the IRF family of transcription factors and mediate production of anti-viral cytokines. During TLR3 and TLR4 signalling by LPS and Poly(I:C) respectively, TRIF is an absolute requirement for IFN-β induction, a process mediated by IRF3 and IRF7 (see section 3.1). However, in pDCs Myd88 acts as the sole adaptor for the activation of IRF7 in response to TLR7/9 signalling (Hemmi et al., 2003b, Diebold et al., 2004). It is thought that autophagy, a conserved process involving lysosomal degradation and recycling of cellular organelles, is involved in delivery of cytoplasmic viral/bacterial nucleic acid to the endosomal TLR7/9 (Lee *et al*., 2007). Upon TLR7/9 activation, Myd88 is recruited to the endosome and initiates the assembly of a specialised signalling complex containing IRAK4, IRAK1, TRAF6 and IRF7 (Kawai *et al*., 2004). TRAF6 E3 ligases activity and IRF7 polyubiquitination is a critical event in IRF7 induction of IFN-α (Kawai *et al*., 2004). Another essential modification for IRF7-induced gene transcription is phosphorylation, which allows IRF7 to dimerise and expose its transactivation domain (Marie *et al*., 2000). In Myd88-dependent signalling IRF7 phosphorylation is mediated by IRAK4, IRAK1 and IKKα as deficiency in any of the kinases results in ablated IFNα upregulation (Yang *et al*., 2005, Uematsu *et al*., 2005, Kim *et al*., 2007a, Hoshino *et al*., 2006). In addition to

type 1 IFN production, pDCs also activate pro-inflammatory cytokines upon infection. This is thought to be facilitated by TRAF6 activation of TAK1 in a similar manner to that displayed in TLR4 and IL-1β signalling (Gilliet *et al*., 2008). It would therefore be easy to speculate that TLR9-Myd88 signalling in other cell types would signal through a similar pathway. However, a surprising observation was discovered when TLR9 activation of type 1 IFNs was dissected in myeloid/conventional DCs (mDCs) and macrophages. Unlike TLR7/9 signalling in pDCs (and TLR3/4 signalling in mDCs), TLR9 activation of antiviral cytokines was independent of IRF7 (and IRF3) in mDCs (Schmitz *et al*., 2007a). Instead TLR9 drives type 1 IFN production through a new signalling axis involving Myd88 and IRF1 highlighting the complexities of Myd88-dependent signalling in both different cell types and different receptor engagements (Negishi *et al*., 2006). In addition, Myd88 facilitates activation of IRF5 in both mDC and pDCs to mediate proinflammatory cytokine production (Takaoka *et al*., 2005).

Taken together, Myd88 dependent signalling is a complex process that is still not completely understood. The ability of the adaptor to recruit differential signalling components to diverse stimuli in different cell populations is only beginning to be uncovered while its place in combination with the other PRRs and the IL-1/18R is still in its infancy. What is known however is that Myd88 is a critical and potent mediator of both pro-inflammatory and anti-viral cytokine production. Given its vital role in host defence signalling, it is not surprising that rare naturally occurring polymorphisms that interfere with TIR adaptor recruitment of the Myddosme confer susceptibility to reoccurring and life-threatening infections (von Bernuth *et al*., 2008, George *et al*., 2011).

1.5.3 Negative Regulation of TLR Signalling

Although TLR detection of infectious microorganisms is essential for host survival, the powerful pro-inflammatory and anti-viral mediators that enable pathogenic elimination can have a profound adverse effect on host homeostasis. As such, TLRs can also be directly involved in the pathogenesis of chronic inflammatory and

autoimmune disease. One of the most serious examples of an inflammatory damage to the host is sepsis. TLR4 recognition of endotoxin from Gram negative bacteria is a major driving force in septic shock (Poltorak *et al*., 1998). This often lethal condition is characterised by an excessive pro-inflammatory cytokine response and the over-production of IL-1β and tumour necrosis factor-α (TNF-α) directly contribute to disease pathology (Remick *et al*., 1990, Hultgren *et al*., 2002, Dinarello, 1997). Recently, it has been found that TLR3 is highly upregulated in the synovium of patients with rheumatoid arthritis (RA) and can detect RNA released from necrotic synovial fluid during disease progression and the subsequent upregulation of cytokines by TLR3 may be a key factor in the inflammatory condition (Brentano *et al*., 2005, Roelofs *et al*., 2005). In addition, raised levels of type 1 IFNs have been observed in systemic lupus erythematosus (SLE) patients and it has been shown to correlate with disease severity (Bengtsson *et al*., 2000). Recently it has been proposed that TLR7/9 activated pDCs are the source of the pathogenic IFN-α in SLE (Blanco *et al*., 2001, Barrat *et al*., 2005, Baccala *et al*., 2013). Taken together, aberrant TLR activation can lead to serious and life threatening conditions and therefore must be subject to tight regulatory control mechanisms after pathogen elimination. In order to control the complex signalling triggered by TLR activation, a number of distinct mechanisms have evolved to limit harmful inflammation (overviewed in Fig. 1.2). Such regulatory mechanisms are often directly activated or transcriptionally upregulated by the signal they function to inhibit.

1.5.3.1 Splice Variants Negatively Regulate TLR signalling

The first line of regulation of TLRs can be seen at the level of the receptors themselves. An alternatively spliced variant of murine TLR4 has been identified as a soluble decoy receptor which contains the ligand binding LRR domain but lacks the intracellular signalling domain and functions as an inhibitor of LPS-mediated pro-inflammatory cytokine production (Iwami *et al*., 2000). Subsequently a similar splice variant has been discovered in humans. The upregulation of this splice variant is deficient in cystic fibrosis patients and is associated with increased NF-κB-induced

TNF-α production, highlighting its potential role in preventing autoimmune disease (Jarešová *et al*., 2007). In addition to TLR4 soluble decoys, a short splice variant of its partner MD-2 has also been discovered and termed MD-2s. MD-2s has been shown to be secreted from the cell and bind TLR4 and compete with full length MD-2 to prevent TLR4 signal transduction (Gray *et al*., 2010, Ohta *et al*., 2004). This method of splice variant decoy inhibition is not restricted to TLR4 signalling as a naturally occurring splice variant of TLR2 (TLR2s) has also been described (LeBouder *et al*., 2003). TLR2s consists of the majority of the extracellular domain of TLR2 and inhibits the receptors functionality by interfering with the recognition of Gram-positive bacteria and supresses pro-inflammatory cytokine induction (Raby *et al*., 2009).

Splice variants of the TIR adaptor proteins have also been discovered. Myd88 short (Myd88s) lacks exon 2 which encodes the inter-domain between the DD and the TIR domain, and can be upregulated by LPS stimulation (Janssens *et al*., 2002). Importantly, Myd88s was shown to negatively regulate IL-1 and TLR4 signalling but not TNF-α induced NF-κB activation. The formation of a Myd88 and Myd88s heterodimer prevents TLR-induced activation of IRAK1 and IRAK4 (Janssens *et al*., 2003, Burns *et al*., 2003). An alternatively spliced form of TRAM which contains TRAMs TIR domain fused to a Golgi dynamics (GOLD) domain termed TRAM adaptor with GOLD domain (TAG) has been shown to specially inhibit TLR4 Myd88 independent signalling. It mediates this function by displacing TRIF from full length TRAM specifically disrupting the TLR4/IRF3 activation pathway (Palsson-McDermott *et al*., 2009). A number of splice variants of the IRAK family members have been discovered. Two splice variants of murine IRAK2, IRAK2c and IRAK2d, have been revealed to lack a DD and function to inhibit TLR activation of NF-κB (Hardy and O'Neill, 2004). In addition, a human splice variant of IRAK1, IRAK1c, also has the ability to block TLR signalling. IRAK1c cannot be phosphorylated by IRAK4, and lacks its catalytic domain but can still associate with Myd88 to prevent downstream signalling. Interestingly, after LPS stimulation, IRAK1 is subject to ubiquitin-dependent degradation while IRAK1c is up regulated

highlighting an auto-inhibitory loop exists for IRAK1 signalling (Rao *et al*., 2005). Splice variants of the TLRs, the TIR adaptors and key signalling intermediates are often upregulated to compete with their full length counterparts to protect the host from excessive inflammation.

1.5.3.2 Transmembrane Receptors as Negative Regulators

In addition to splice variant TLR inhibition, a number of transmembrane proteins have been characterised that negatively regulate inflammatory signalling. A novel member of the IL-1R superfamily, single Ig IL-1R-related molecule (SIGIRR) or TIR-8 is a single transmembrane TIR domain containing protein that cannot activate NF-κB (Thomassen *et al*., 1999). It was found that SIGIRR acts as a negative regulator of Myd88-dependent induction of pro-inflammatory cytokines (Wald *et al*., 2003). SIGIRRs mechanism of inhibition has only been partially elucidated. While SIGIRRs attenuation of IL-1R signalling requires both extracellular and intracellular domains to be intact, its intracellular TIR domain alone is sufficient to inhibit TLR4 activity and it has been proposed to act as a molecular trap to prevent IRAK and TRAF6 downstream signalling (Qin *et al*., 2005). Interestingly, although its expression is not readily upregulated by pro-inflammatory stimuli, it is highly restricted to cells of the epithelium and may act to prevent excessive inflammation in the gastrointestinal tract, a major interface between the host and microorganisms (Garlanda *et al*., 2004).

The TNF-related apoptosis-inducing ligand (TRAIL) receptor (TRAILR) also functions as a negative regulator of TLR signalling. Unlike SIGIRR, TRAILR does not contain a TIR domain and as the name implies is a member of the TNFR superfamily (Wu *et al*., 1999). TRAILR-deficient cells displayed increased cytokine expression upon TLR3 and TLR4 stimulation, stimuli which also induced TRAILR expression. TRAIL was showed to play a physiological role in regulating the immune response as TRAIL-deficient mice were more resistant to and showed enhanced clearance of mouse cytomegalovirus infection, which correlated with increased levels of pro-inflammatory cytokines and IFN-β. Mechanistically,

TRAILR signalling inhibits IκBα degradation preventing sustained NF-κB nuclear translocation (Diehl *et al*., 2004). Furthermore, TRAILR has been shown to directly protect against chronic inflammation and tumorigenesis in mouse models highlighting the key role transmembrane negative regulators of TLR signalling exert on host protection (Finnberg *et al*., 2008).

1.5.3.3 TIR Domain Containing Proteins Regulate TLR Signalling

There is no doubt that TIR adaptor containing proteins play fundamental roles in the instigation of TLR signalling pathways. However the discovery of independent TIR domain containing proteins that negatively regulate TLR signalling led to a re-evaluation of their respective roles in innate immunity.

The fifth TIR adaptor protein discovered was Sterile-α and Armadillo motif containing protein (SARM) (Mink *et al*., 2001). SARM is the most ancient of the TIR adaptors and has a direct orthologue in *Caenorhabditis elegans* termed TIR-1 (Couillault *et al*., 2004). TIR-1 was shown to play a vital role in anti-fungal defence by acting upstream of the worm orthologue of p38. Surprisingly however, unlike the other adaptor proteins, overexpressed human SARM is incapable of activating NF-κB (Liberati *et al*., 2004). In human cells, SARM has been shown to acts as a specific inhibitor of TLR3 and TLR4 activation of TRIF dependent cytokine induction in a TIR domain dependent manner. LPS induces SARM protein expression and SARM directly interacts with TRIF suggesting SARMs involvement in a negative feed-back loop. Interestingly, generation of SARM-deficient mice showed no phenotypic differences to wild type animals upon TLR stimulation, with SARM expression being restricted to the central nervous system (CNS) (Kim *et al*., 2007b). In addition, a recent study concluded that Bunyavirus infection, a major cause of encephalitis, results in a RIG-I and mitochondrial antiviral signalling protein (MAVS) signalling pathway leading to the upregulation and induction of SARM-mediated neuronal cell death (Mukherjee *et al*., 2013). Therefore, it is still unclear the exact role of SARM in TLR signalling and further investigation into

redundancy and species specific roles of the TIR adaptor are required to elucidate its innate immune functionality.

Recently, B-cell adapter for PI3K (BCAP) has been discovered to contain a previously unknown TIR domain (Troutman *et al*., 2012). As the name implies BCAP was originally discovered to link B cell receptor engagement to PI3K pathway induction (Okada *et al*., 2000, Inabe and Kurosaki, 2002). Two groups simultaneously discovered its similar role in TLR signalling (Troutman *et al*., 2012, Ni *et al*., 2012). Troutman *et al.* ascribed its ability to link TLR signalling to PI3K induction as being TIR dependent. Genetic disruption of BCAP leads to enhanced production of pro-inflammatory cytokines but has no effect on NF-κB and MAPK pathway activation (Ni *et al*., 2012, Troutman *et al*., 2012). Instead, BCAP is a critical factor in TLR phosphorylation of protein kinase B (also known as AKT) which in turn negatively regulates pro-inflammatory cytokine production. Although this process is still incompletely understood, phosphorylated AKT has been shown to upregulate the microRNA let-7e which decreases TLR4 transcript stability and downregulate miR-155 that targets suppressor of cytokine signalling 1 (SOCS1), a known TLR inhibiting protein (Androulidaki *et al*., 2009). In addition, AKT can phosphorylate glycogen synthase kinase 3b (GSK3b) inducing its degradation to suppress optimal pro-inflammatory IL-12 production (Ohtani *et al*., 2008). Although the exact mechanism remains to be defined, the TIR containing protein is a novel and potent inhibitor of TLR signalling.

As outlined above, Myd88 is the key TIR adaptor for all TLR signalling pathways with the exception of TLR3. It was therefore surprising that Myd88 was shown to play a negative regulatory role in TLR3-induced corneal inflammation in a murine disease model (Johnson *et al*., 2008). In another murine model, Myd88-deficiency during viral myocarditis by Coxsackievirus B3 proved to be protective. This correlated with increased type 1 IFNs present in the cardiac tissue of the infected Myd88-deficient animals in conjunction with increased IRF3 activation (Fuse *et al*., 2005). Investigation into the mechanism by which the adaptor negatively regulated TLR3 signalling revealed that Myd88 specifically targeted the activation of IRF3 by

IKKε but not TBK1 (Siednienko *et al.*, 2011). Similar to Myd88, TIRAP has also been shown to have regulatory functions in TRIF-dependent signalling pathways. Initial observations made in TIRAP-deficient cells showed an increase in TLR3-induced expression of IL-6 (Kenny *et al.*, 2009). Furthermore, TLR3 activation of IFN-β by IRF7 was found to be inhibited by TIRAP associating with IRF7 and blocking its activation of the positive regulatory domain I/III of the IFN-β promoter (Siednienko *et al.*, 2010). Interestingly, TRIF has recently been shown to potentially act as a negative regulator of Myd88-dependent induction of IL-12p70 and IL-6 in the TLR4 and TLR7 pathways of murine dendritic cells (Seregin *et al.*, 2011). Although no mechanistic investigation has yet been conducted, it raises the interesting possibility of the TIR adaptors antagonising differential TLR cascades in order to fine tune and regulate the innate immune response.

1.5.3.4 Negative Regulation of TLR Signalling by Post Translational Modifications

Although splice variants, membrane domain proteins and the TIR adaptors themselves can regulate TLR signalling, vast arrays of enzymatic intracellular proteins have also been discovered to curtail excessive inflammatory signals by direct post-translational modifications. It is clear from the above that phosphorylation represents a critical step in the activation of the innate immune response and it is not surprising therefore that dephosphorylation events are often vital in inflammatory resolution.

Recessive mutations in the murine gene $Hcph^{me}$ (or $Ptpn6^{me}$) that encodes the tyrosine kinase phosphatase Src homology protein tyrosine phosphatase (SHP-1) exhibit a condition known as “moth-eaten (*me/me*)” (Shultz *et al.*, 1993). These mice are characterised by severe defects in hematopoiesis resulting in autoimmune symptoms, including immune complex deposition and auto-antibody production, with early mortality due to pneumonitis and other inflammatory disorders (Sidman *et al.*, 1984, Greiner *et al.*, 1986). Interestingly, another mutation in the *Ptpn6* gene known as spin, also elicits a chronic inflammatory condition that can be rescued if

mice are kept in a germfree environment. In addition, individual deficiencies in Myd88, IRAK4 and IL-1R also prevent the autoimmune disorders associated with this mutation indicating that SHP-1 plays an essential role in control of TLR-mediated inflammation (Croker *et al.*, 2008). SHP-1 was found to bind to the immunoreceptor tyrosine-based inhibition motif (ITIM) in the IRAK1 kinase domain whereby it prevents IRAK1 phosphorylation and its catalytic activity (An *et al.*, 2008).

Members of the MAPK family can also be regulated by reversible phosphorylation events of conserved threonine and tyrosine residues in their tripeptide TXY signature motifs (Robinson and Cobb, 1997). A key mediator of MAPK dephosphorylation is the dual specificity (phosphor-tyrosine and phospho-serine/phospho-threonine) phosphatase MAP kinase phosphatase-1 (MKP-1) (Sun *et al.*, 1993). TLR activation of MAPKs leads to the transcriptional upregualtion of MKP-1 and protein stability is maintained by ERK activity (Chen *et al.*, 2002, Chi *et al.*, 2006). Mice genetically deficient in MKP-1 are characterised by enhanced TLR4 induction of pro-inflammatory cytokines and increased susceptibility to endotoxic shock. The increases in TNF-α and IL-6 are associated with prolonged p38 and JNK phosphorylation and AP-1 transcriptional activity (Chi *et al.*, 2006, Zhao *et al.*, 2006, Salojin *et al.*, 2006). Interestingly, acetylation of MKP-1 during TLR signalling has been shown to increase MKP-1 interaction with p38 and increase p38 dephosphorylation (Cao *et al.*, 2008). Therefore, MAPK regulation by dephosphorylation is vital to defend against an excessive pro-inflammatory response.

Studies revealing MKP-1 acetylation were surprising as acetylation by histone acetyltransferases (HATs) and histone deacetylases (HDACs) has generally been associated with regulating gene expression by modifying histone proteins (Allfrey *et al.*, 1964, Peterson, 2002). TLR4 activation leads to the recruitment of HDAC1 by the CREB related transcription factor activating transcription factor-3 (ATF-3) to NF-κB responsive genes and this causes deactelyation of associated histones resulting in the closure of chromatin and repression of gene expression (Gilchrist *et al.*, 2006). Interestingly however, an increasing body of evidence has placed

acetylation as an important mediator of NF-κB regulation. Phosphorylation of the NF-κB family subunit p65 allows the recruitment of the nuclear cofactors p300/CBP and P/CAF that enable histone modification to allow gene transcription (Sheppard *et al*., 1999, Zhong *et al*., 1998, Sterner and Berger, 2000). Furthermore, p65 is the direct substrate for acetylation, and this post-translational modification prevents nuclear p65 from re-associating with *de vovo* IκBα. Upon deacetylation by HDAC3, p65 binds to IκBα and is exported from the nucleus via a chromosomal region maintenance-1 (CRM-1) dependent motif thereby limiting p65 transcriptional activity (Chen *et al*., 2001).

A recent study has highlighted the complexity of acetylation events in pro-inflammatory gene expression. During LPS-induced tolerance, a number of pro-inflammatory genes but not antimicrobial mediators are desensitised to further gene transcription upon subsequent exposure to signal (Foster *et al*., 2007). This is mediated in part by the p50 subunit of NF-κB (that alone lacks transactivation potential) undergoing homo-dimerisation and subsequent blockage of transcriptional active NF-κB dimers to their shared gene responsive promoter sites (Ziegler-Heitbrock *et al*., 1994, Schmitz and Baeuerle, 1991, Carmody *et al*., 2007). Upon TLR4 activation, p50 undergoes deacetylation and forms a repressorsome complex with nuclear receptor corepressor (NcoR), HDAC1, and HDAC3. This stable complex occupies genes containing an NF-κB binding motif (termed tolerisable genes) and renders them unresponsive to further LPS stimulation while allowing genes not containing this motif to be reactivated by subsequent stimulation (Yan *et al*., 2012). Taken together, alterations in phosphorylation and acetylation represent significant regulatory mechanisms in TLR signalling.

1.6 Ubiquitination and TLR Regulation

1.6.1 The Ubiquitination Pathway

Ubiquitination represents one of the most important regulatory systems in biology and is extensively used to orchestrate and regulate the complex signalling proteins utilised in the innate immune response (Bhoj and Chen, 2009). Ubiquitin is a highly conserved 76 amino-acid polypeptide that is ubiquitously expressed in eukaryotic cells (Goldstein *et al*., 1975, Wilkinson and Audhya, 1981). The ubiquitination process involves a 3 step enzymatic cascade involving a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase protein (E3) (Hershko *et al*., 1983). In humans there are two ubiquitin E1 enzymes, approximately forty to fifty E2 enzymes and hundreds of E3 ubiquitin ligases (Jiang and Chen, 2012). The E1 enzyme initiates the cascade by using ATP to adenylate ubiquitin at its C-terminal glycine residue. The sulfhydryl group of the catalytic cysteine in the active site of the E1 then attacks the adenylate to form a thioester bond between E1 and ubiquitin allowing AMP to be released. The ubiquitin bound E1 can then readily transfer the activated ubiquitin to the active cysteine site of the E2 enzyme through a transthioesterification. The cascade is complete when an E3 ubiquitin ligase binds to the E2-ubiquitin complex and a substrate protein to facilitate the transfer of ubiquitin via a covalent isopeptide linkage between the carboxyl-terminal glycine of ubiquitin and the ε-amino group of a lysine residue on the target protein (Strieter and Korasick, 2011, Herrmann *et al*., 2007, Pickart and Eddins, 2004). The E3 ligases mediate substrate specificity and have been a major focus of research in TLR signalling. Two distinct groups of E3 ligases have been distinguished. The RING domain E3 ligases are characterised by an octet of cysteine and histidine residues that constitute a zinc binding domain (Lorick *et al*., 1999). Mutations of these cysteine residues interfere with the zinc binding domain and E2 ligase interaction and often result in a loss in E3 ligase activity (Itahana *et al*., 2007, Deshaies and Joazeiro, 2009). RING domain E3 ligases are not thought to transfer the ubiquitin from E2 onto an active site on themselves and instead function by

positioning the reactive ubiquitin-E2 thioester bond in close proximity to the nucleophilic lysine residue of the substrate protein and may initiate conformation changes to aid ubiquitin substrate transfer (Deshaies and Joazeiro, 2009). The other group of E3 ligases are known as the homologous to E6-activating protein (E6-AP) carboxy terminus (HECT) domain containing enzymes. These E3 ligases contain an invariant cysteine residue that accepts the ubiquitin molecule directly from the E2 enzyme in which ubiquitin is thioesterified to the HECT domain, as is the case for the archetypical member E6-AP (Scheffner *et al.*, 1995). The ubiquitin is then transferred from this intermediate to the substrate by the HECT E3 directly.

Ubiquitination of a protein can occur at a single lysine in a process known as monoubiquitination or ubiquitin chains can be formed as E2 and E3 enzymes catalyse the formation of a covalent isopeptide bond between a carboxyl group of one ubiquitin glycine to the amino group of another ubiquitin's lysine residue, a process known as polyubiquitination (Terrell *et al.*, 1998, Chau *et al.*, 1989). In addition, a protein may be subjected to multiple ubiquitination events on different lysine residues simultaneously a process termed multi-ubiquitination (Petroski and Deshaies, 2003). Monoubiquitin events have mainly been associated with receptor internalisation and endocytosis, trafficking and gene silencing (Terrell *et al.*, 1998, Endoh *et al.*, 2012, Wu *et al.*, 2011). In regards to polyubiquitin chain formation, the ubiquitin molecule contains 7 lysine residues (K6, K11, K27, K29, K33, K48, and K63) and an N-terminal methionine residue (M1) all of which can act as a ubiquitin acceptor site (Xu *et al.*, 2009, Kirisako *et al.*, 2006). While K48-linked polyubiquitination targets proteins for proteasomal degradation, K63-linked chains function in non-proteolytic events that modify the proteins signalling functionality. The roles of the other lysine linkages (K6, K11, K27, K29 and K33) and linear ubiquitination (M1) remain to be fully elucidated but are thought to function in both proteasomal dependent and independent processes. Like phosphorylation, ubiquitination is a reversible modification and serves as a key regulatory consequence of signal transduction. This process is facilitated by approximately 100 deubiquitinating (DUB) enzymes, cysteine metalloproteases that cleave ubiquitin

chains from protein substrates or their degradation remnants (Sun, 2008). Ubiquitin-specific proteases (USPs) are the largest family of DUBs and contain two short well conserved motifs, Cys and His boxes that include residues critical for catalysis. The different USPs also contain motifs and sequences outside this domain which may confer substrate specificity (Nijman *et al.*, 2005). As the E3 ubiquitin ligases and their antagonising DUB enzymes represent a major regulatory control mechanism, it is not surprising they have been heavily implicated in the control of TLR signalling pathways.

1.6.2 Polyubiquitination, a Key Regulator of TLR Signalling

The critical nature of polyubiquitin chain formation in facilitating TLR signalling is self-evident. TRAF6 dependent K63-linked ubiquitination mediates IKK-induced phosphorylation of IκBα and triggers its K48-linked degradation, allowing NF-κB to translocate to the nucleus and induce gene transcription. However, in conjugation with its positive role in TLR signal propagation, polyubiquintation events play key roles in the negative regulation of the inflammatory response.

TLR4 and TLR9 activation potently upregulates the expression of the SOCS box containing protein SOCS1, a protein thought to possess E3 ligase like activity (Crespo *et al.*, 2000, Dalpke *et al.*, 2001). The SOCS box is characterised by its ability to bind elongin B, elongin C, cullin-5 and RING-box-2 (RBX2), which recruits E2 ubiquitin transferase to mediate ubiquitin conjugation activity (Kamura *et al.*, 2004). Interestingly, SOCS1-deficient mice display hyper-responsive LPS-induced cytokine production and increased susceptibility to endotoxin-mediated septic shock (Kinjyo et al., 2002, Nakagawa et al., 2002b). SOCS1 mediates its negative regulation of TLR4 during a negative feedback loop that is thought to involve the ubquitination and proteasomal degradation of a number of key signalling components of the pathway. During TLR4 signalling Bruton's tyrosine kinase (Btk) phosphorylates TIRAP facilitating the recruitment of SOCS1 triggering TIRAP polyubiquintation on lysines 15 and 16. This modification then targets the TIR

adaptor for proteasome-mediated degradation (Mansell *et al.*, 2006). As SOCS1 can also inhibit TLR9 signalling, a TLR that does not utilise TIRAP, it must target other proteins in this signalling cascade (Nakagawa et al., 2002a). Recently, SOCS1 has also been implicated in the direct polyubiquintation of the NF-κB subunit p65 (Strebovsky *et al.*, 2011). In this model, SOCS1 translocates to the nucleus and interacts with p65 via the former's SH2 domain. SOCS1 was shown to preferentially bind p65 but not a transcriptionally inactive mutant form. In turn, SOCS1 p65 interaction leads to SOCS box dependent ubiquitination and degradation of p65 to curtail pro-inflammatory cytokine production.

Ubiquitin dependent proteasomal regulation of the innate immune response is not restricted to NF-κB and pro-inflammatory signalling. Recently, RTA-associated ubiquitin ligase (RAUL) has been implicated in the direct catalysis of K48-linked polyubiquitination of the antiviral transcription factors IRF3 and IRF7 leading to their proteasomal degradation (Yu and Hayward, 2010). RAUL is a HECT domain containing E3 ligases that under resting conditions is thought to undergo auto-ubiquitination-mediated self-degradation. Interestingly, during antiviral signalling by TLR3 or RIG-I, RAUL associates with a DUB enzyme known as USP7 leading to the removal of K48-linked chains from the E3 ligase. This newly stabilised protein can then in turn mediated polyubiquitination of its target transcription factors thereby limiting a potentially harmful type 1 IFN response. This mechanism of action highlights the complex dynamics of ubiquitin events that control the innate immune response. Furthermore, as IRF7 requires K63- linked ubiquitination for activation and induction of gene expression, an interplay between the different ubiquitin linkages possibly mediated by additional DUBs and/or E3 ligases is crucial in the type 1 IFN response (Ning *et al.*, 2008a). USP7 has also recently been shown to regulate NF-κB-mediated transcriptional events. RNA interference (RNAi) studies specifically targeting USP7 revealed it had a functional role in maintaining TLR-induced pro-inflammatory cytokine production. USP7 however had no effect on upstream NF-κB signalling intermediates or on p65 nuclear translocation. USP7 functions by deubiquitinating promoter bound p65, preventing its proteasomal

degradation and therefore allowing maintained pro-inflammatory gene expression (Colleran *et al*., 2013). These two studies highlight an intriguing scenario whereby a single DUB enzyme can function as a positive regulator of the pro-inflammatory but negatively regulator of the anti-viral response, indicating it may play a key determining role in the innate immune response mounted to a specific pathogen threat.

A20 is a zinc RING domain containing E3 ligase that can also be classed as a cysteine protease DUB due to a catalytic deubiquitination domain at its N-terminus (Opipari *et al*., 1990, Wertz *et al*., 2004). A20 is rapidly upregulated during LPS signalling and in turn negatively regulates TLR activation of pro-inflammatory cytokine production (Cooper *et al*., 1996, Boone *et al*., 2004, O'Reilly and Moynagh, 2003). Upon TLR4 activation, A20 is recruited to TRAF6 and facilitates the removal of K63-linked chains preventing its ability to bind the TAK1 TAB complex (Boone *et al*., 2004, Heyninck and Beyaert, 1999). A20 also has the ability to potently inhibit IRF3 transcriptional activity (Saitoh *et al*., 2005). It is thought that A20 in combination with its adaptor protein Tax1-binding protein 1 (TAX1BP1) binds the non-canonical IKKs, TBK1 and IKKε, and removes K63-linked ubiquitin chains that aid in IRF3 phosphorylation to restrict the antiviral response (Parvatiyar *et al*., 2010). A20 has also been implicated in the negative regulation of IRF7 during Epstein-Barr virus (EBV) oncoprotein latent membrane protein (LMP) 1-mediated activation. A20 binds to IRF7 via its DUB domain and mediates K63-linked deubiquitination of the transcription factor correlating with decreased antiviral transcriptional activity (Ning and Pagano, 2010).

Conceptually, it would be easy to say that K63 ubiquitination propagates TLR downstream inflammatory signalling and deubiquitination serves as a key regulator in curtailing this response. Emerging evidence in the field however is highlighting a more complex regulatory system is at play. TRAF6, in addition to conjugating K63-linked ubiquitin chains to activate the NF-κB pathway, has also been shown to directly polyubiquitinate AKT of the PI3K pathway. K63 modified AKT can then relocate to the plasma membrane whereby it becomes phosphorylated and activated

(Yang *et al*., 2009b). Although the authors of this study did not examine its effect on the inflammatory response, it is known that the TIR domain containing protein BCAP negatively regulates TLR signalling at the membrane via AKT recruitment and downstream signal activation and one could speculate that this may represent a novel regulatory mechanism at play in TLR signalling. In any case, as the sheer number of E3 ligases identified in eukaryotic signalling has now outpaced that of the kinase family, further work is warranted and required to fully access the role polyubiquitination plays in the innate immune response (Deshaies and Joazeiro, 2009).

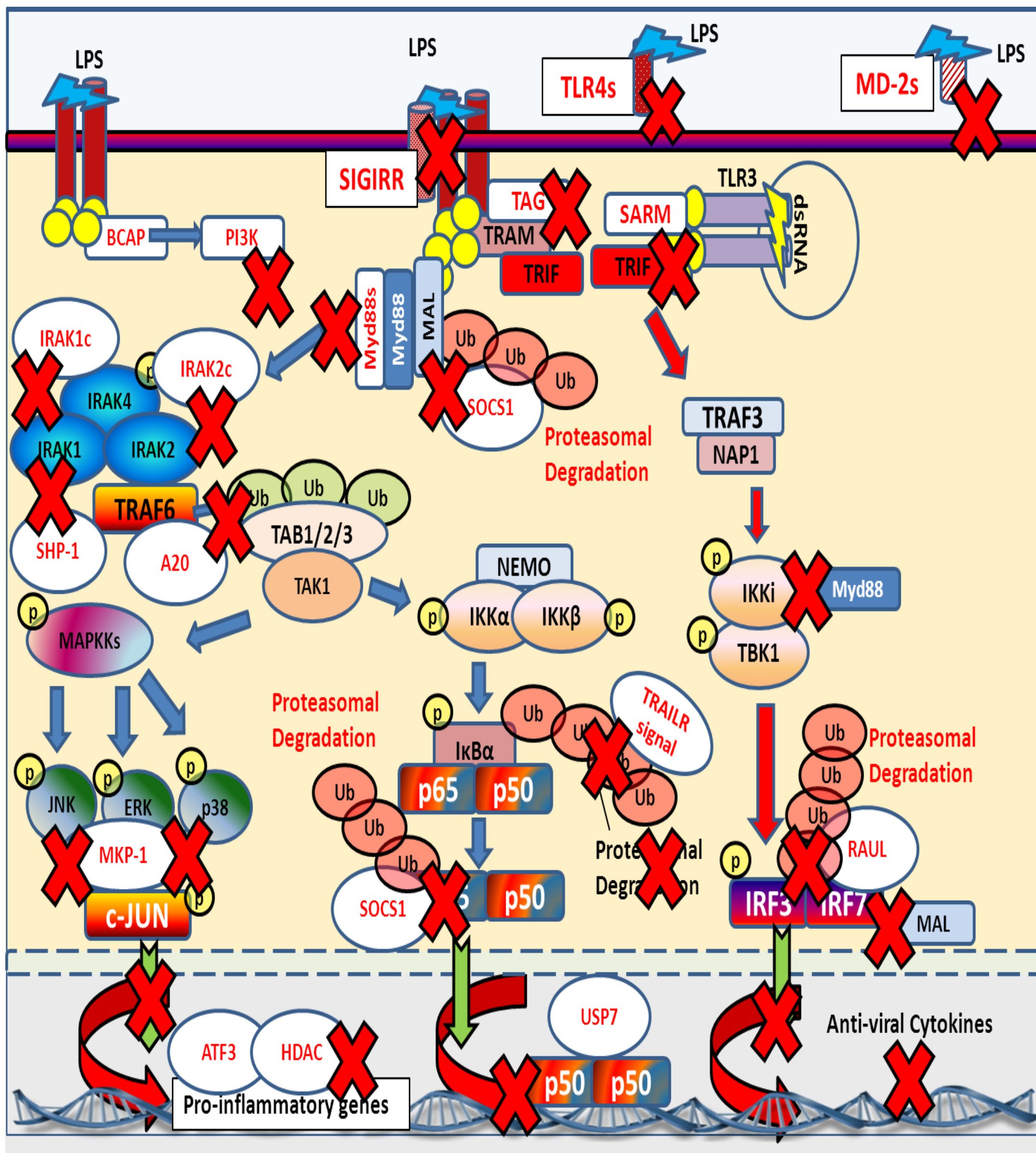


Fig. 1.2 Negative regulation of TLR signalling

See section 1.5.3 for detailed description of negative regulation of TLR signalling by splice variant, transmembrane receptors, the TIR adaptors, phosphatases and acetylation modifications. See section 1.6.2 for detailed description of negative regulation by ubiquitination. White circles with red labels identify negative regulators of TLR signalling. Red Xs represent where in the pathway is targeted for regulation.

1.7 The Pellino Family

It is clear from the above that ubiquitination is a central regulator of TLR signalling. The recent discovery of a mammalian family of closely related E3 ubiquitin ligases known as the Pellino proteins, that show strong homology to the Pelle (IRAK) interacting protein in the *Drosphila* Toll pathway, has focused research effort on their roles in TLR signalling. Pellino was first identified in 1999 in a yeast two-hybrid screen and shortly afterwards 3 mammalian family members were uncovered, genetically annotated *Peli1*, *Peli2* and *Peli3* (Grosshans et al., 1999, Rich et al., 2000, Resch et al., 2001, Jensen and Whitehead, 2003c). The human Pellino proteins, Pellino1, Pellino2 and two splice variants of Pellino3 known as Pellino3a and Pellino3b are highly conserved and encoded on chromosomes 2, 14 and 11 respectively. Pellino1 and Pellino2 are most similar in size and identity as the Pellino3 proteins have an additional 27 amino acid leader sequence n their N terminus. Pellino3a consists of 7 exons however Pellino3b is missing exon2 leading to an in frame deletion of 24 amino acids. Similarly, murine Pellino1, Pellino2 and Pellino3 are also highly conserved; one noticeable difference however is that Pellino3 only has one isoform and no splice variants in the mouse genome. In addition murine Pellino1, Pellino2 and Pellino3 are mapped to chromosomes 11, 14 and 19 respectively. Pellino proteins have also been highly selected throughout evolution with human Pellino1 sharing 40% aa homology to *Caenorhabditis elegans* Pellino (Schauvliege *et al*., 2007). This high degree of conservation between the different species and the selection of 3 isoforms in most mammals suggests the Pellino proteins serve an important biological purpose(s) (see Fig. 1.3 for sequence alignments and Table 1.2 for percentage sequence identity).

At the time of their discovery in mammals, the Pellino proteins were shown to be able to bind IRAK1, IRAK4, TAK1 and TRAF6 suggesting that Pellino proteins may function in TLR signal transduction (Butler et al., 2005, Jiang et al., 2003a, Strelow et al., 2003b). As no functional or catalytic domains had been ascribed for the Pellino proteins, they were thought to act as mere signalling scaffolds (Jensen

and Whitehead, 2003c). However the presence of a RING-like domain (CHC2CHC2) in the Pellino proteins C terminus indicated that they may possess E3 ubiquitin ligase activity. Overexpression studies showed that all Pellino proteins could bind to IRAK1 and facilitate its polyubiquitination (Schauvliege *et al*., 2006). Subsequent studies showed that the Pellino proteins could directly catalyse K63-linked polyubiquitin chains of IRAK1 *in vitro* and that they are in fact E3 ubiquitin ligases (Butler *et al*., 2007). Furthermore, the Pellino proteins E3 ligase activity was shown to be under direct control of IRAK1 and IRAK4 phosphorylation. In addition, Pellino1 was shown to be able to associate with a number of E2 ubiquitin conjugating enzymes and mediate not only K63, but also K48 and K11-linked chain formation *in vitro* suggesting Pellino proteins can mediate diverse cellular signalling events (Ordureau *et al*., 2008). Intriguingly, the RING-like domain of the Pellino proteins does not mediate IRAK binding. Resolution of the X-ray crystal structure of Pellino2 lacking its RING-like domain revealed the presence of a cryptic N terminal forkhead associated (FHA) domain (Lin *et al*., 2008), a module known to confer recognition and binding of phosphor-serine-theronine residues on interacting proteins. The Pellino2 FHA domain was found to be responsible for interaction with phosphorylated IRAK. Interestingly, this FHA domain is flanked by two atypical wing appendages, the function of which are still unknown (Lin *et al*., 2008). A viral form of Pellino has been identified in the genome of a member of the Entomopoxvirinae (poxviruses of insects) family, specifically, *Melanoplus sanguinipes* entomopoxvirus (MsEPV), a virus that infects the North American grasshopper (Rich *et al*., 2000, Afonso *et al*., 1999). Viral Pellino (vPellino) does not contain a RING-like domain or the wing appendages around its FHA domain. Interestingly vPellino was shown to be able to bind to phosphorylated IRAK but not induce its polyubiquitination and inhibit both Toll and TLR-mediated anti-microbial/inflammatory responses (Griffin *et al*., 2011). As viruses have evolved to target key innate immune signalling complexes and only advantageous viral proteins are retained, the selection of a viral Pellino protein highlights the potential importance the Pellino proteins must play in the innate immune response.

```
hPeli1      1 ---------------------------MFSPDQENH--PSKAPVKYGELIVLG-------
mPeli1      1 ---------------------------MFSPDQENH--PSKAPVKYGELIVLG-------
hPeli2      1 ---------------------------MFSPGQEEHCAPNKEPVKYGELVVLG-------
mPeli2      1 ---------------------------MFSPGQEEPSAPNKEPVKYGELVVLG-------
hPeli3a     1 MVLEGNPEVGSPRTSDLQHRGNKGSCVLSSPGEDAQ--PGEEPIKYGELIVLGCCEEGGE
hPeli3b     1 MVLEGNPEVGSPRTSDLQHRGNKGSCVLSSPGEDAQ--PGEEPIKYGELIVLG-------
mPeli3      1 MVLEGNPDVGSPRTSDLQHPGSQGSCILSCPGEEAL--AGEEPIKYGELIVLG-------
dPellino    1 ---------------MVKRTDGTESPILAEDGGDGH---DKPRLRYGELVILG-------

hPeli1     25 -----------------YNGSLPNGDRGRRKSRFALFKRPKANGVKPSTVHIACTPQAAK
mPeli1     25 -----------------YNGSLPNGDRGRRKSRFALFKRPKANGVKPSTVHIACTPQAAK
hPeli2     27 -----------------YNGALPNGDRGRRKSRFALYKRPKANGVKPSTVHVISTPQASK
mPeli2     27 -----------------YNGALPNGDRGRRKSRFALYKRTYASGVKPSTIHMVSTPQASK
hPeli3a    59 ETEAQRGEVTGPRAHSCYNGCLASGDKGRRRSRLALSRRSHANGVKPDVMHHISTPLVSK
hPeli3b    52 -----------------YNGCLASGDKGRRRSRLALSRRSHANGVKPDVMHHISTPLVSK
mPeli3     25 -----------------YNGCLASGDKGRRRSRLALSRRPHANGVKPDVMHHISTPLVSK
dPellino   36 -----------------YNGYLPQGDRGRRRSKFVLHKRTEASGVKRSKHYIVQSPQTSK

hPeli1     68 AISNKDQHSISYTLSRAQTVVVEYTHDSNTDMFQIGRSTESPIDFVVTDTVPGSQSNSDT
mPeli1     68 AISNKDQHSISYTLSRAQTVVVEYTHDSNTDMFQIGRSTESPIDFVVTDTVPGSQSNSDT
hPeli2     70 AISCKGQHSISYTLSRNQTVVVEYTHDKDTDMFQVGRSTESPIDFVVTDTISGSQNTDEA
mPeli2     70 AISSRGHHSISYTLSRSQTVVVEYTHDKDTDMFQVGRSTESPIDFVVTDTVSGGQNE-DA
hPeli3a   119 ALSNRGQHSISYTLSRSHSVIVEYTHDSDTDMFQIGRSTENMIDFVVTDTSPGGGAA-EG
hPeli3b    95 ALSNRGQHSISYTLSRSHSVIVEYTHDSDTDMFQIGRSTENMIDFVVTDTSPGGGAA-EG
mPeli3     68 ALSNRGQHSISFTLSRSHSVIVEYTHDSDKDMFQIGRSTENMIDFVVTDTSPGGGAT-EG
dPellino   79 AILDANQHSISYTLSRNQAVIVEYKEDTETDMFQVGRSSESPIDFVVMDTLPGDKK--DA

hPeli1    128 QSVQSTISRFACRIICERNPPFTARIYAAGFDSSKNIFLGEKAAKWKTSDGQMDGLTTNG
mPeli1    128 QSVQSTISRFACRIICERSPPFTARIYAAGFDSSKNIFLGEKAAKWKTSDGQMDGLTTNG
hPeli2    130 QITQSTISRFACRIVCDRNEPYTARIFAAGFDSSKNIFLGEKAAKWKNPDGHMDGLTTNG
mPeli2    129 QITQSTISRFACRIVCDRNEPYTARIFAAGFDSSKNIFLGEKAAKWKNPDGHMDGLTTNG
hPeli3a   178 PSAQSTISRYACRILCDRRPPYTARIYAAGFDASSNIFLGERAAKWRTPDGLMDGLTTNG
hPeli3b   154 PSAQSTISRYACRILCDRRPPYTARIYAAGFDASSNIFLGERAAKWRTPDGLMDGLTTNG
mPeli3    127 PSAQSTISRYACRILCDRRPPYTARIYAAGFDASSNIFLGERAAKWRTPDGLMDGLTTNG
dPellino  137 KVMQSTISRFACRILVNRCEPAKARIFAAGFDSSRNIFLGEKATKWQDN-VEIDGLTTNG

hPeli1    188 VLVMHPRNGFTED-SKPGIWREISVCGNVFSLRETRSAQQRGKMVEIETNQLQDGSLIDL
mPeli1    188 VLVMHPRNGFTED-SKPGIWREISVCGNVFSLRETRSAQQRGKMVEIETNQLQDGSLIDL
hPeli2    190 VLVMHPRGGFTEE-SQPGVWREISVCGDVYTLRETRSAQQRGKLVESETNVLQDGSLIDL
mPeli2    189 VLVMHPQGGFTEE-SQPGVWREISVCGDVYTLRETRSAQQRGKLVESETNVLQDGSLIDL
hPeli3a   238 VLVMHPAGGFSED-SAPGVWREISVCGNVYTLRDSRSAQQRGKLVENESNVLQDGSLIDL
hPeli3b   214 VLVMHPAGGFSED-SAPGVWREISVCGNVYTLRDSRSAQQRGKLVENESNVLQDGSLIDL
mPeli3    187 VLVMHPAGGFSED-SAPGVWREISVCGNVYTLRDSRSAQQRGKLVENESNVLQDGSLIDL
dPellino  196 VLIMHPKGSFCGGNAKCGLWRECSVGGDVFSLRESRSAQQKGQPIYDECNILQDGTLIDL

hPeli1    247 CGATLLWRTAEGLSHTPTVKHLEALRQEINAARPQCPVGFNTLAFPSMKRKD-VVDEKQP
mPeli1    247 CGATLLWRTAEGLSHTPTVKHLEALRQEINAARPQCPVGFNTLAFPSMKRKD-VVDEKQP
hPeli2    249 CGATLLWRTADGLFHTPTQKHIEALRQEINAARPQCPVGLNTLAFPSINRKE-VVEEKQP
mPeli2    248 CGATLLWRTADGLFHAPTQKHIEALRQEINAARPQCPVGLNTLAFPSINRKE-VVEEKQP
hPeli3a   297 CGATLLWRTPAGLLRAPTLKQLEAQRQEANAARPQCPVGLSTLAFPSPARGRTAPDKQQP
hPeli3b   273 CGATLLWRTPAGLLRAPTLKQLEAQRQEANAARPQCPVGLSTLAFPSPARGRTAPDKQQP
mPeli3    246 CGATLLWRTPAGLLRAPTLKQLEAQRQEANAARPQCPVGLSTLAFPSPARGRTAPDKQQP
dPellino  256 CGATLLWRSAEGLQHSPTKHDLEKLIDAINAGRPQCPVGLNTLVIPRKVNI--GDQVNQP

hPeli1    306 WVYLNCGHVHGYHNWGNKEERDGKDRECPMCRSVGPYVPLWLGCEAGFYVDAGPPTHAFS
mPeli1    306 WVYLNCGHVHGYHNWGNKEERDGKDRECPMCRSVGPYVPLWLGCEAGFYVDAGPPTHAFS
hPeli2    308 WAYLSCGHVHGYHNWGHRSDTEANERECPMCRTVGPYVPLWLGCEAGFYVDAGPPTHAFT
mPeli2    307 WAYLSCGHVHGYHSWGHRSDTEANERECPMCRTVGPYVPLWLGCEAGFYVDAGPPTHAFT
hPeli3a   357 WVYVRCGHVHGYHGWGCRRERGPQERECPLCRLVGPYVPLWLGQEAGLCLDPGPPSHAFA
hPeli3b   333 WVYVRCGHVHGYHGWGCRRERGPQERECPLCRLVGPYVPLWLGQEAGLCLDPGPPSHAFA
mPeli3    306 WVYVRCGHVHGYHGWGCRREQGPQERECPLCRLVGPYVPLWLGQEAGLCLDPGPPSHAFA
dPellino  314 YVYLNCGHVQGHHDWGQDENTG--ARRCPMCLELGPVVTLCMGLEPAFYVDVGAPTYAFN

hPeli1    366 PCGHVCSEKTTAYWSQIPLPHGTHTFHAACPFCAHQLAGEQGYIRLIFQGPLD
mPeli1    366 PCGHVCSEKTTAYWSQIPLPHGTHTFHAACPFCAHQLAGEQGYIRLIFQGPLD
hPeli2    368 PCGHVCSEKSAKYWSQIPLPHGTHAFHAACPFCATQLVGEQNCIKLIFQGPID
mPeli2    367 PCGHVCSEKSAKYWSQIPLPHGTHAFHAACPFCATQLVGEQNCIKLIFQGPVD
hPeli3a   417 PCGHVCSEKTARYWAQTPLPHGTHAFHAACPFCGAWLTGEHGCVRLIFQGPLD
hPeli3b   393 PCGHVCSEKTARYWAQTPLPHGTHAFHAACPFCGAWLTGEHGCVRLIFQGPLD
mPeli3    366 PCGHVCSEKTARYWAQTPLPHGTHAFHAACPFCGAWLTGELGCVRLIFQGPLD
dPellino  372 PCGHMATEKTVKYWANVEIPHGTNGFQAVCPFCATPLDGATGYIKLIFQDNLD
```

Fig. 1.3 Amino Acid Sequence Alignment of the Pellino proteins

Amino acid (aa) sequence alignment for human (h), murine (m) and drosophila (d) Pellino (Peli) proteins. Black shading indicates identical aa and grey indicates similar aa. Analysis conducted with CLUSTALW and Boxshade software.

	hPeli1	**mPeli1**	**hPeli2**	**mPeli2**	**hPeli3a**	**hPeli3b**	**mPeli3**	**dPellino**
hPeli1	100.00	99.76	81.82	79.86	70.50	70.50	69.78	60.49
mPeli1	99.76	100.00	81.58	79.62	70.50	70.50	69.78	60.49
hPeli2	81.82	81.58	100.00	95.23	71.22	71.22	70.74	60.24
mPeli2	79.86	79.62	95.23	71.22	71.22	100.00	70.50	60.49
hPeli3a	70.50	70.50	71.22	71.22	100.00	100.00	96.63	52.25
hPeli3b	70.50	70.50	71.22	71.22	100.00	100.00	96.63	52.25
mPeli3	69.78	69.78	70.74	70.50	96.63	96.63	100.00	51.77
dPellino	60.49	60.49	60.24	60.49	52.25	52.25	51.77	100.00

Table 1.2 Pellino Proteins Percentage Sequence Identity

Table shows the percentage sequence identity between human (h), mouse (m) and fly (d) Pellino (Peli) proteins. Analysis conducted with CLUSTALW software.

1.7.1 Pellino1

Initial investigations into the role of Pellino1 during the immune response were solely based on overexpression and RNAi approaches. Such studies concluded that Pellino1 played an important role in both IL-1R and TLR signalling cascades by the formation of polyubiquitin chains on IRAK molecules to facilitate downstream activation of the NF-κB pathway (Jiang *et al*., 2003a, Schauvliege *et al*., 2006, Butler *et al*., 2007). Such studies were further complemented by the finding that the inhibitory properties of TGF-β and the TGF-β family member Bone morphogenetic protein-4 (BMP-4) on IL-1R and TLR signalling were mediated at the Pellino1 level. TGF-β signalling was shown to activate SMAD6 which could associate with Pellino1 and prevent the formation of the Pellino1-IRAK1-IRAK4-TRAF6 signalling complex thereby abrogating NF-κB activation (Choi *et al*., 2006, Lee *et al*., 2010).

Although such approaches all indicated that Pellino1 functions at the level of IRAK signalling, the generation of Pellino1-deficient mice proved otherwise as Pellino1 was not required to propagate IL-1R signalling. Instead, Pellino1 was essential for TRIF dependent activation of the IKKs and NF-κB and the subsequent pro-inflammatory cytokine response. Importantly, this investigation revealed that Pellino1 interacted with and facilitated RIP1 polyubiquitination, a modification crucial for TRIF-dependent signalling (Chang *et al*., 2009). Interestingly however, Pellino1 was also required for TLR9 signalling in B cells, a pathway that does not require TRIF and RIP1 activity indicating other functional roles for the Pellino during TLR signalling (Zhang *et al*., 2011, Hoebe *et al*., 2003). A number of years later the same group discovered that Pellino1 was required to prevent autoimmunity by inhibiting T cell activation as Pellino1-deficient mice spontaneously develop multi-organ inflammation and auto-antibody production due to hyper active T cells that are refractory to regulatory T cell suppression (Chang *et al*., 2011). Surprisingly, Pellino1 was found to function in the K48-linked polyubiquitination and proteasomal degradation of c-REL, a late phase induced NF-κB family subunit in T cells

responsible for transcription of genes required for T cell activation, differentiation and prevention of anergy (Chang *et al.*, 2011). More recently, a third study from this group demonstrated that Pellino1 also played important roles in CNS inflammation. Pellino1 was required for MAPK activation in microglial cells. Pellino1 was shown to bind to TRAF3, a potent inhibitor of MAPK activation, leading to its K48-linked ubquitination and degradation allowing optimal induction of pro-inflammatory and chemoattractant cytokines. Furthermore, this had striking physiological consequences in experimental autoimmune encephalomyelitis whereby Pellino1-deficient mice do not display recruitment of pathogenic T cells into the CNS and are highly protected from disease onset and progression (Xiao *et al.*, 2013).

The generation of a specific knock-in mouse model in which the wild type Pellino1 protein was replaced with a Pellino1^{F397A} mutant thought to be devoid of E3 ligase activity revealed a further role for Pellino1 in innate immunity (Enesa *et al.*, 2012). Pellino1^{F397A} mice display a reduction in type 1 IFN production in response to dsRNA viruses and ligands with reduced binding of IRF3 to the IFN-β (Enesa *et al.*, 2012). Interestingly, TLR3 induction of RIP1 polyubiquitination and MAPK activation is not unaffected in this mouse model. Although the reason for this is unclear, it is possible that the single aa substitution in the knock-in mouse may not fully abolish the proteins E3 ligase activity or the protein may aid in the recruitment of another catalytically active mediator.

In addition to the functional roles Pellino1 plays in the innate immune response, recent investigations into the upstream activators of Pellino1 that govern its downstream activities have been explored using newly synthesised pharmacological kinase inhibitors. During Il-1R signalling IRAK1 is the major kinase responsible for Pellino1 phosphorylation, while during TLR signalling the IKK related kinases, TBK1 and IKKε, mediate phosphorylation (Goh *et al.*, 2012, Smith *et al.*, 2011). Although additional insight is required to fully delineate Pellino1 functionality, the generation of Pellino1-deficient mice was highly informative in understanding the importance of this proteins role in the complexities of the immune response.

1.7.2 Pellino2

Although the role of Pellino1 in the innate immune response has been extensively investigated, the functional role of Pellino2 in innate immunity remains ambiguous. As was the case for Pellino1, Pellino2 was first reported to interact with and be phosphorylated by IRAK1 and IRAK4 (Strelow et al., 2003b). However, unlike Pellino1, overexpression of Pellino2 does not induce the transcriptional activity of the NF-κB reporter construct (Strelow et al., 2003a). In contrast, overexpression and RNAi knockdown of murine Pellino2 indicated that the protein may function in IL-1R and TLR4 but not TNF-α activation of the IL-8 promoter (Yu *et al*., 2002). Further overexpression studies demonstrated that Pellino2 could also interact with TRAF6 and TAK1 and stimulate the kinase activity of JNK, culminating in increased activity of the transcription factors AP-1 and Elk-1 (Jensen and Whitehead, 2003a). However, another study concluded that Pellino2 acted as a bridging partner for Bcl-10 in a LPS-induced signalling complex including Bcl-10-MALT1-TRAF6-TAK1 for NF-κB activation and had no role in AP-1 or Elk-1 activity (Liu *et al*., 2004, Dong *et al*., 2006). To date, the most comprehensive report into the function of Pellino2 in innate immunity indicates that the E3 ligase may be required for phosphorylation of the MAPKs JNK and ERK to maintain pro-inflammatory transcript stability (Kim *et al*., 2012). Although the role of Pellino2 in innate immune signalling is somewhat controversial, a few reports indicating it may play a role in human health are emerging. Recently, a microarray analyses of 128 genes involved in the TLR signalling pathway were performed in nephrectomy samples of patients with chronic allograft nephropathy and acute rejection. Of all the genes screened Pellino2, IL-8 and the E2 ubiquitin conjugating enzyme E2-UBE2V1 were the only significantly upregulated genes indicating a potential role in inflammation induced transplant damage and rejection (Nogueira *et al*., 2009). A genome wide association study also mapped a number of single nucleotide polymorphisms (SNP) to chromosome 14q21 that correlated with decreased susceptibility to bladder cancer. One SNP, rs398652 was found to occur in the intergenic space just before *Peli2* and the authors of the study suggested that a SNP in *Peli2* may be associated with

decreased inflammation and decreased cancer risk (Gu *et al*., 2011). However, like the basic research into Pellino2's functionality *in vitro* these clinical observations require more effort in order to define Pellino2's role in inflammation. The generation of a Pellino2-deficient animal will no doubt aid in delineation of the E3 ligases physiological role in the immune response.

1.7.3 Pellino3

Pellino3 transcript is present in most human tissue with highest expression levels in the heart, brain and testes (Jensen and Whitehead, 2003b). Furthermore, it was shown that Pellino3 could also interact with IRAK1, TRAF6 and TAK1 upon IL-1R signalling but its overexpression failed to activate NF-κB reporter constructs. Instead, Pellino3 was thought to activate MAPK kinase activity and trigger increased transcriptional activity of c-JUN and Elk-1 (Jensen and Whitehead, 2003b). However, as was the case for Pellino2, there have been contrasting reports on the function of Pellino3. An additional study indicated that knockdown of Pellino3 by siRNA augments TAK1 dependent signalling and IL-8 production (Xiao *et al*., 2008). In support of the initial finding that Pellino3 may act in the MAPK pathways, overexpression of Pellino3 was shown to induce p38 phosphorylation. In addition, knockdown of Pellino3 with siRNA led to a decrease in Il-1β activation of p38. The ability of Pellino3 to activate p38 was reliant on TRAF6 and TAK1 functionality (Butler *et al*., 2005). Further studies into Pellino3's role in p38 activation revealed that Pellino3 was required in a signalling axis for TLR4 activation of the transcription factor CREB in a TRAF6 and p38 dependent manner. Interestingly, mutation of cysteine residues in the Pellino3 RING-like domain rendered Pellino3 signal transduction ineffective (Mellett *et al*., 2011). The most current report on Pellino3's function indicates that Pellino3 and IRAK1 are activated by oxidized low-density lipoprotein (oxLDL) that accumulates in atherosclerotic lesions. Upon macrophage ingestion of oxLDL, they transform into lipid laden foam cells (Conway and Kinter, 2006). It was recently shown that oxLDL treated macrophages have a reduced capacity to trigger IFN-β upon LPS stimulation. Mechanistically, it has been

proposed that oxLDL activated IRAK1 and Pellino3 induce TANK modification leading to inactivation of TBK1 and reduced phosphorylation of IRF3 (Tzieply *et al*., 2012).

Taken together, Pellino3 has been implicated in NF-κB, MAPK/AP-1 and the IRF signalling pathways. However, an exact mechanistic understanding of how Pellino3 functions in TLR signalling and what physiologically relevant role it holds is still largely unknown. In order to address this deficit in innate immune signalling knowledge, this studies aims to elucidate the physiological role of Pellino3 in TLR signalling.

1.8 Project Aims

The aims of this thesis were:

- To employ Pellino3-deficient mice and RNAi knockdown approaches to define the function of Pellino3 in TLR signalling.
- To delineate the mechanistic basis underlying the regulatory role(s) of Pellino3 in TLR signalling.

Chapter 2:

Materials and Methods

2.1 Materials

2.1.1 Reagents

Reagents	**Supplier**
7-AAD Viability Staining Solution	eBiosciences
Agar	Sigma
Agarose	Promega
Ampicillin	Sigma
APS	Sigma
Bovine serum albumin (BSA)	Sigma
TRAF6 siRNA	Ambion
GAPDH siRNA	Ambion
Pellino3 shRNA	Sigma
Control shRNA	Sigma
Bradford reagent dye	Bio-Rad
Bromophenol blue	Sigma
CHAPS	Sigma
Chloroform	Sigma
CLO97	Invivogen
Coomassie Blue (G250)	Sigma
CpG type A ODN 1585	Invivogen
CpG type B ODN 1666	Invivogen

DirectPCR Lysis Reagent (Tail)	Viagen Biotech
DMEM (Dulbecco's Modified Eagle's medium)	Invitrogen
DMSO (dimethyl sulfoxid)	Sigma
DNA ladder & Loading dye	Promega
dNTPs (deoxyribonucleotide triphosphates)	Promega
DTT (dithiothreitol)	Sigma
E.coli-TOP10 competent cells	Invitrogen
EDTA (ethylenediaminetetra-acetic acid)	Sigma
Ethanol	Sigma
Ethidium bromide	Sigma
FBS (fetal bovine serum)	Invitrogen
Flagellin	Invivogen
Glycerol	Sigma
Glycine	Sigma
GoTaq® polymerase	Promega
GoTaq® Green Master Mix	Promega
HEPES	Sigma
Hydrochloric acid (HCl)	Merck
Hydrogen peroxide solution (H_2O_2)	Sigma
Igepal	Sigma
Isopropanol	Sigma
Kanamycin	Sigma

Lipofectamine 2000	Invitrogen
LPS	Enzo Life Sciences
Lysogeny broth (LB)	Sigma
Magnesium Chloride	Sigma
Methanol	BDH
Molecular Biology Grade Water (RNase and DNase Free)	Sigma
NF-κB IRDye Labelled Oligonucleotides	Licor Biosciences
NF-κB non-labelled Oligonucleotides	MWG
OptiMEM	Invitrogen
Pam_2CSK4	Invivogen
Pam_3CSK4	Invivogen
PBS (Phosphate buffered saline)	Oxoid
Penicillin / Streptomycin / Glutamine	Invitrogen
PMSF (phenylmethylsulfonyl fluoride)	Sigma
Poly(I:C)	Invivogen
PRD I/III non-labelled Oligonucleotides	MWG
PRD I/III IRDye Labelled Oligonucleotides	MWG
Prestained molecular weight marker	Invitrogen
Protease inhibitor cocktail	Roche
Protein A/G-agarose	Santa Cruz
Proteinase K	Qiagen
Protogel	National Diagnostics

Random primers	Invitrogen
Recombinant murine FLT3L	Miltenyi Biotec
Recombinant murine GM-CSF	Immunotools
Recombinant murine IFN-α4	PBL Interferon Source
Recombinant murine IFN-β	Peprotech
RNase Zap	Ambion
SDS (sodium dodecyl sulphate)	Sigma
siRNA Scrambled Control	Ambion
siRNA TRAF6 specific	Ambion
Skim milk powder	Sigma
Sodium chloride (NaCl)	Sigma
Sodium hydroxide (NaOH)	Sigma
Sodium orthovanadate (Na_3VO_4)	Sigma
Sodium Phosphate (Na_3PO_4)	Sigma
Sulphuric acid (H_2SO_4)	Sigma
Synthetic oligonucleotides	MWG Biotech
TEMED (N, N, N′, N′-tetramethylethylenediamine)	Sigma
TMB (3, 3’, 5, 5’-Tetramethylbenzidine liquid substrate)	Sigma
Tris-base	Sigma
Tris-HCl	Sigma
Trition-X-100	Sigma
Trypsin/EDTA	Invitrogen

Tween-20	Sigma
Zymosan	Invivogen
pMSCV (Murine STEM Cell Virus)	Clotech
Blasticidin	Invivogen
Zeocin	Invivogen
2-Mercaptoethanol	Gibeco
Ponceau s	Sigma
Acetic Acid	Fisher
WesternBright ECL HRP Substrate	Advansta

2.1.2 Kits

Kits	**Supplier**
BioScript (Reverse Transcriptase) 1st-Strand cDNA Synthesis Kit	Bioline
EMSA (Electophoretic Mobility Shift Assay) Buffer Kit	Licor Biosciences
Mouse IFN-α Platinum ELISA Kit	Ebiosciences
Mouse IL-6 ELISA Kit	R&D Systems
Mouse IP-10 ELISA Kit	Peprotech
Mouse TNF-α ELISA Kit	R&D Systems
Plasmacytoid Dendritic Cell Isolation Kit II, mouse	Miltenyi Biotec
Plasmid Plus Midi Kit	Qiagen
SensiMix SYBR No-ROX Kit	Bioline

2.1.3 Antibodies

2.1.3.1 Antibodies for Immunoblotting

Primary Antibodies	Dilution Factor	Diluent	Supplier
ERK	1:1000	5% BSA TBST	Cell Signalling
Fibrillarin	1:1000	5% Milk TBST	Cell Signalling
FLAG M2	1:1000	5% Milk TBST	Sigma
IKK-β	1:1000	5% BSA TBST	Cell Signalling
IRF3	1:500	5% Milk TBST	Santa Cruz
IRF7 (Mouse)	1:200	5% Milk TBST	Santa Cruz
IRF7 (Rabbit)	1:1000	5% Milk TBST	Abcam
IκB-α	1:1000	5% BSA TBST	Santa Cruz
JNK	1:1000	5% BSA TBST	Cell Signalling
Myc-Tag (9B11)	1:2000	5% Milk TBST	Cell Signalling
p38	1:1000	5% BSA TBST	Cell Signalling
p65	1:200	5% Milk TBST	Santa Cruz
Peli3	1:200	5% Milk TBST	GenScript
Phosho-p38	1:1000	5% BSA TBST	Cell Signalling
Phospho-ERK	1:1000	5% BSA TBST	Cell Signalling
Phospho-IκB-α	1:500	5% Milk TBST	Cell Signalling
Phospho-JNK	1:1000	5% BSA TBST	Cell Signalling
Phospho-cJUN	1:1000	5% BSA TBST	Cell Signalling
Phospho-IKK-α/β	1:750	5% BSA TBST	Cell Signalling
Phospho-IRF3	1:1000	5% BSA TBST	Cell Signalling
Phospho-p65	1:1000	5% BSA TBST	Cell Signalling
Phospho-Serine	1:500	5% BSA TBST	Millipore
Phospho-STAT1	1:1000	5% BSA TBST	Cell Signalling
Phospho-STAT3	1:1000	5% BSA TBST	Cell Signalling
Phospho-TBK1	1:1000	5% BSA TBST	Cell Signalling
TBK1	1:1000	5% Milk TBST	Cell Signalling
TRAF6	1:200	5% Milk TBST	Santa Cruz

Ubiquitin	1:200	5% Milk TBST	Santa Cruz
β-actin	1:5000	5% Milk TBST	Sigma

Secondary Licor Antibodies	**Dilution Factor**	**Diluent**	**Supplier**
IRDye 680 Goat Anti-Mouse	1:5000	5% Milk TBST	Licor Biosciences
IRDye 800CW Goat Anti-Rabbit	1:5000	5% Milk TBST	Licor Biosciences

Secondary ECL Antibodies	**Dilution Factor**	**Diluent**	**Supplier**
Anti-mouse HRP	1:1000	5% Milk TBST	Cell Signalling
Anti-rabbit HRP	1:4000	5% Milk TBST	Promega

2.1.3.2 Antibodies for IFN-β ELISA

Antibody	**Function**	**Dilution Factor**	**Supplier**
Rat anti-Mouse IFN-β	Capture	1:1000	Santa Cruz
Rabbit anti-Mouse IFN-β	Detection	1:2000	PBL Interferon Source
anti-Rabbit HRP	Reporter	1:2000	Promega

2.1.3.3 Antibodies for Flow Cytometry

Flow Cytometry Antibodies	**Isotype Controls**	**Supplier**
anti-Mouse CD11c-APC	anti-Armenian Hamster IgG-APC	Ebiosciences
anti-Mouse CD11b-PE	anti-Rat IgG2bκ-PE	Ebiosciences
Anti-Mouse/Human CD45R-FITC	anti-Rat IgG2aκ-FITC	Ebiosciences

2.1.4 Cell Lines

Cells	**Description**
HEK293-TLR3	Human embryonic kidney cells, stably expressing human Toll-like Receptor 3

U373-MG	Human glioblastoma-astrocytoma, epithelial-like cell line
B16-Blue™ IFN-α/β Cells	Cells allow the quantitative detection of bioactive murine type 1 IFNs via measurement of the IFNAR activation of JAK/STAT/ISGF3 pathway.
WT iBMDM	Immortalised BMDM derived from WT mice
TRIF$^{-/-}$ iBMDM	Immortalised BMDM derived from TRIF$^{-/-}$ mice
TBK1$^{-/-}$ iBMDM	Immortalised BMDM derived from TBK1$^{-/-}$ mice
MAVS$^{-/-}$ iBMDM	Immortalised BMDM derived from MAVS$^{-/-}$ mice

2.1.5 Buffers

Buffer	**Composition**
Blocking Buffer for Immunoblotting	TBS, 0.1% (v/v) Tween-20 with 5 % (w/v) non-fat dry Milk
Blocking Buffer for ELISA	PBS, 1% (w/v) BSA, Filtered Sterile 0.02µm
Cell Lysis Buffer and CoIP Lysis Buffer	50 mM HEPES pH 7.5, 10% (v/v) Glycerol, 0.5% (w/v) CHAPS, 0.5% (v/v) Triton-X-100, 250 mM NaCl, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM EDTA and protease inhibitor mixture cocktail
Laemmli sample buffer	62.5 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, 0.7 M β-mercaptoethanol and 0.001% (w/v) bromophenol blue
Nuclear Fraction Buffer-A	10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1% (v/v) Igepal , 0.5 mM DTT and 0.5 mM PMSF
Nuclear Fraction Buffer-B	20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (w/v) glycerol and 0.5 mM PMSF
Nuclear Fraction Buffer-C	10 mM HEPES pH 7.9, 50 mM KCl, 0.2 mM EDTA,

	20% (w/v) glycerol, 0.5 mM PMSF and 0.5 mM DTT
PBS (Phosphate buffered saline)	2.7 mM KCl, 1.5mM KH_2PO_4, 137 mM NaCl and 8 mM Na_2HPO_4, pH 7.4
Reagent diluent for ELISA	0.1% (w/v) BSA, 0.05% (v/v) Tween in TBS
RIPA Lysis Buffer	50 mM Tris-HCl pH 7.4, 1% (v/v) Igepal, 150 mM NaCl, 0.5% (w/v) Sodium Deoxycholate, 1 mM EDTA, 0.1% (w/v) SDS 1 mM Na_3VO_4, 1 mM PMSF and protease inhibitor cocktail
SDS running Buffer	25 mM Tris, 192 mM glycine, 0.1% SDS.
TAE (Tris-acetate-EDTA) Buffer	40 mM Tris base, 0.1% (v/v) glacial acetic acid, 1 mM EDTA
TBE (Tris-Borate-EDTA) Buffer	90 mM Tris Base, 90 mM Boric Acid, 2 mM EDTA
TBS (Tris buffered saline)	25 mM Tris, pH7.4, containing 0.14M NaCl.
TE (Tris-EDTA) Buffer	10 mM Tris-HCl, 1 mM EDTA pH 8.0
Transfer Buffer Ponceau Stain	25 mM Tris, 192 mM glycine, 20% (v/v) methanol 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid

2.1.6 Primer Sequences

2.1.6.1 Primers for Quantitative Real-Time PCR

Targeted Gene	Sense Primer Sequence 5'-3' Antisense Primer Sequence 5'-3'
Human HPRT	AGCTTGCTGGTGAAAAGGAC TTATAGTCAAGGGCATATCC
Human IFN-β	AACTGCAACCTTTCGAAGCC TGTCGCCTACTACCTGTTGTGC
Human IL-6	AGCCACTCACCTCTTCAGAACGAA CAGTGCCTCTTTGCTGCTTTCACA
Murine HPRT	GCTTGCTGGTGAAAAGGACCTCTCGAAG CCCTGAAGTACTCATTATAGTCAAGGGCAT
Murine IFN-α4	GGCTTGACACTCCTGGTACAAATGAG

	CAGCACATTGGCAGAGGAAGACAG
Murine IFN-β	GGAGATGACGGAGAAGATGC CCCAGTGCTGGAGAAATTGT
Murine IL-6	ACAACCACGGCCTTCCCTAC TCCACGATTTCCCGAGAACA
Murine IP-10	GACGGTCCGCTGCAACTG GCTTCCCTATGGCCCTCATT
Murine Pellino3	ACATGCCAACGGAGTGAAGC AGCGGCCAATCTGGAACAT
Murine TNF-α	CATCTTCTCAAAATTCGAGTGACAA TGGGAGTAGACAAGGTACAACCC

2.1.6.2 Primers for PCR targeting Genomic DNA for genotyping

Allele Targeted	**Sense Primer Sequence 5'-3'** **Antisense Primer Sequence 5'-3'**
HPRT	GCTTGCTGGTGAAAAGGACCTCTCGAAG CCCTGAAGTACTCATTATAGTCAAGGGCAT
WT and Recombined Peli3 Allele **Primers a + b**	CCCAACATAGGTGTTTCCTCTCC GTGCATACACATTCATGCAAGC
WT Peli3 Allele Only **Primers c + d**	GACACGTGTGGAGATAATGAGG ACCCAGGCACAAGTCAAGC

2.1.7 Animals

Pellino3-deficient mice and their wild-type littermates were bred at the Bioresource Unit of the National University of Ireland Maynooth. Genotyping was performed by PCR analysis of genomic DNA from ear punches as described in 2.2.1 and genotypes were reconfirmed after the completion of experiments. The mice were housed under specific pathogen free (SPF) conditions in individually ventilated cages (IVC), enriched with cardboard housing and nesting material. The animals were kept at room temperature (22 – 24°C) in a 12hr light/dark cycle (lights on at 8:00 a.m.) with *ad libitum* access to food and water. All mice were used under the

guidelines of the Irish Department of Health, and all procedures were approved by the research ethics committee of the National University of Ireland Maynooth.

2.1.8 Gifts

2.1.8.1 Cells

WT and *Traf6*$^{-/-}$ MEFs: Prof. Andrew Bowie (Trinity College Dublin, Dublin, Ireland)

HEK293 stably expressing TLR3: Prof. Douglas Golenbock (The University of Massachusetts Medical School, Worcestor, Massachusettes 01605, USA)

U373-MG: Dr. Sinead Miggin (National University of Ireland Maynooth, Co. Kildare, Ireland)

WT and *MAVS*$^{-/-}$ iBMDM: Dr. Sinead Miggin (National University of Ireland Maynooth, Co. Kildare, Ireland)

WT, *TRIF*$^{-/-}$ and *TBK1*$^{-/-}$ iBMDM: Prof. Katherine Fitzgerald (The University of Massachusetts Medical School, Worcestor, Massachusettes 01605, USA)

L929 cells expressing M-CSF and J558 cells expressing GM-CSF: Prof. Luke O'Neill (Trinity College Dublin, Dublin, Ireland)

2.1.8.2 Constructs

FLAG-tagged WT TRAF6, TRAF6$^{(C70A)}$ and TRAF6$^{(K124A)}$: Prof. Andrew Bowie (Trinity College Dublin, Dublin, Ireland)

FLAG-tagged IRF7: Dr. Marion Butler (National University of Ireland Maynooth, Co. Kildare, Ireland)

Myc-tagged human WT Pellino3: Dr. Marion Butler (National University of Maynooth, Co. Kildare, Ireland

Myc-tagged human Pellino3 Ring Mutant$^{(C360A/C363A)}$: Dr. Lisa Tang (National University of Maynooth, Co. Kildare, Ireland

Myc-tagged murine WT Pellino3 and Pellino3 Ring Mutant$^{(C360A/C363A)}$: Dr. Shuo Yang (National University of Maynooth, Co. Kildare, Ireland

2.1.8.3 Live Viruses

Encephalomyocarditis virus (EMCV): Dr. Jakub Siednienko (Polish Academy of Sciences, Wrocław, Poland)

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Culturing of Bone Marrow Derived Macrophages (BMDMs)

For the generation of BMDMs, gender and age matched WT and *Peli3*$^{-/-}$ mice (8– 12 weeks) were selected and sacrificed by cervical dislocation. To prevent contamination, mice were placed in a laminar flow hood and sterilised using 70% ethanol and all further work was conducted using aseptic technique. The femur and tibia of the mice were removed and bone marrow was isolated by flushing the bones with a 27¾ gauge needle filled with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS), penicillin G (100 μg/ml) and streptomycin (100 μg/ml). Cells were pelleted by centrifugation at 600 gravitation (g) for 6 minutes (min). The cell pellet was subject to red blood cell lysis (RBL) using 1 ml of Sigma RBL buffer for 1 min. 25 ml of DMEM was added to terminate lysis and cells were again centrifuged at 600 gravitation "g" for 6 min. Bone marrow cells were resuspended in 10 ml DMDM and counted using a haemocytometer. Cells from WT and *Peli3*$^{-/-}$ were equalised for number and seeded at $2x10^6$ cells/ml (25 ml) in a $T175^2$ culture flask. Conditioned media (CM) from

L929 cells that express Macrophage Colony Stimulating Factor (M-CSF) was added to a final concentration of 20% (v/v). The primary cells were maintained in a 37°C humidified atmosphere with 5% CO_2. After 3 days media was gently removed to avoid detachment of the adherent macrophage differentiating cells. Fresh media was added containing 20% (v/v) M-CSF. The removed media containing non-adhered cells were subjected to centrifugation at 600 g for 6 min. Cells were re-suspended in fresh media and placed in a new $T175^2$ culture flask in the presence of 20% (v/v) M-CSF. Cells were incubated for another 3-4 days until fully differentiated (as assessed by cell morphology). The highly adherent BMDMs were isolated from culture flasks by cell scraping and seeded in DMEM without M-CSF for experimental use.

2.2.1.2. Culturing of Bone Marrow Derived Dendritic Cell (BMDC)

For the generation of BMDCs, gender and age matched WT and *Peli3*$^{-/-}$ mice (8 – 12 weeks) were sacrificed by cervical dislocation. To prevent contamination, mice were placed in a laminar flow hood and sterilised using 70% ethanol and all further work was conducted following aseptic technique. The femur and tibia of the mice were removed and the bone marrow was isolated by flushing the bones with a 27¾ gauge needle filled with Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% (v/v) heat inactivated FBS, GlutaMAX™ (200 mM), penicillin G (100 µg/ml), streptomycin (100 µg/ml) and 2-Mercaptoethanol (55 µM). Cells were pelleted by centrifugation at 600 g for 6 min. The cell pellet was subject to RBL using 1 ml of Sigma RBL buffer for 1 min. 25 ml of DMEM was added to terminate lysis and cells were again centrifuged at 600 g for 6 min. Bone marrow cells were resuspended in 10 ml RPMI and counted using a haemocytometer. Cells numbers between WT and *Peli3*$^{-/-}$ were equalised and seeded at $1x10^6$ cells/ml (40 ml) in a $T175^2$ culture flask. CM from J558 cells expressing Granulocyte-macrophage colony-stimulating factor (GM-CSF) was added to a final concentration of 20% (v/v). The primary cells were maintained in a 37°C humidified atmosphere with 5% CO_2. After 3 days the media containing the non-adherent cells was removed and centrifuged at 600 g for 6 min. The semi-adherent cells in the $T175^2$ flask were scrapped in PBS and also subjected to centrifugation. The cell pellets were combined

and counted using a haemocytometer. Cells numbers from WT and *Peli3*$^{-/-}$ mice were equalised and seeded at 1x10^6 cells/ml (40 ml) in 2 T175^2 culture flasks in RPMI with 20% (v/v) GM-CSF. After a further 3 days, the above culture step was repeated. The non-adherent cells constituting the BMDCs, were isolated and seeded for experiments in the absence of GM-CSF or allowed to be cultured for a further 3 days in media containing GM-CSF.

2.2.1.3 Culturing of Bone Marrow derived Plasmacytoid DC (pDC)

pDC generation was achieved by following the protocol outlined in 2.2.1.2 with minor modifications. The culture media used was RPMI supplemented with 10% (v/v) heat inactivated FBS, GlutaMAX™ (200 mM), penicillin G (100 µg/ml), streptomycin (100 µg/ml), 2-Mercaptoethanol (55 µM), 1% (v/v) MEM non-essential amino acids, Sodium pyruvate (1 mM) and HEPES (10 mM). In order to drive the progenitor cells to pDC lineage, recombinant murine FMS-related tyrosine kinase 3 ligand (Flt3L) (100 µg/ml) was added at each culture step. As pDC generation resulted in a heterogeneous suspension cell population, pDC cells were enriched prior to experimental use using the Plasmacytoid Dendritic Cell Isolation Kit II from Miltenyi Biotech as per manufactures instructions.

2.2.1.4 Culturing of Murine Embryonic Fibroblasts (MEFs)

Peli3$^{+/-}$ mice were interbred to produce *Peli3*$^{+/+}$ and *Peli3*$^{-/-}$ embryos. Mouse breeder pairs were set up in the afternoon with one male being set up with two or three 7-8 week old females. Females were checked for copulatory plugs the following morning. If present, this was marked as day +0.5. Females, with copulatory plugs, were removed from the male's breeder cages. The remaining females were checked on a daily basis.

At +13.5 days, pregnant mice were euthanized by cervical dislocation. The uterus (containing the embryos) was cut free from the mouse and placed in a sterile petri dish. Embryos were removed from the uterus one at a time in sterile PBS under sterile conditions in a laminar flow hood. The head and all of the soft tissues from

each embryo were removed so that only the carcasses were left. Embryo carcasses were minced using small scissors or scalpel, and placed in a 50 ml sterile tube containing 1.5 ml PBS. Trypsin-EDTA solution (1% w/v) was added and tissue was mechanically agitated using a sterile 5 ml pipette. The mixture was then incubated at 37ºC for 8 min and again mechanically agitated and returned to incubation for a further 8 min. DMEM (15 ml) supplemented with 10% (v/v) heat inactivated FBS, penicillin G (100 µg/ml) and streptomycin (100 µg/ml) was added and cells were added to a T75^2 culture vessel. The MEF cells were passaged every 3-4 days or until they were confluent. DNA samples were collected from discarded head or soft tissue for genotyping.

2.2.1.5 Culturing of B16-Blue™ IFN-α/β Cells

The murine type 1 IFNs sensor cell line B16-Blue IFN-α/β were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat inactivated FBS, GlutaMAX™ (200 mM), penicillin G (100 µg/ml) and streptomycin (100 µg/ml). Zeocin™ (100 µg/ml) was used to select for expression of the stably integrated cassette encoding the Sh ble gene and embryonic alkaline phosphatase (SEAP) reporter gene under the control of the IFN-α/β-inducible ISG54 promoter. Cell lines were maintained in a 37°C humidified atmosphere with 5% CO_2. Cells were passaged every 2-3 days at approximately 80% confluence using 1% (w/v) Trypsin/EDTA solution in PBS. Cells were cultured for up to 25 passages before being discarded to prevent genetic instability accumulation.

2.2.1.6 Culturing of HEK293-TLR3 and U373 Cells

HEKT293 cells stably expressing TLR3 and U373 cells were cultured in DMEM supplemented with 10% (v/v) heat inactivated FBS, penicillin G (100 µg/ml) and streptomycin (100 µg/ml). Blasticidin (10 µg/ml) was used to select for expression of the stably integrated cassette encoding the TLR3 gene. Cell lines were maintained in a 37°C humidified atmosphere with 5% CO_2. Cells were passaged every 2-3 days at approximately 80% confluence using 1% (w/v) Trypsin/EDTA solution in PBS. Cells were cultured for up to 25 passages before being discarded to prevent genetic instability accumulation.

2.2.1.7 Culturing of Immortalised Bone Marrow Derived Macrophages

Immortalised Bone Marrow derived Macrophages (iBMDM) from WT, *TRIF*$^{-/-}$, *TBK1*$^{-/-}$ and *MAVS*$^{-/-}$ were cultured in RPMI supplemented with 10% (v/v) heat inactivated FBS, penicillin G (100 µg/ml) and streptomycin (100 µg/ml). Cell lines were maintained in a 37°C humidified atmosphere with 5% CO_2. The highly adherent cells were passaged every 2-3 days at approximately 80% confluence using a cell scraper in PBS. Cells were cultured for up to 25 passages before being discarded to prevent genetic instability accumulation.

2.2.1.8 Culturing of L929 Cells and M-CSF Media Production

L929 cells were cultured in DMEM supplemented with 10% (v/v) heat inactivated FBS, penicillin G (100 µg/ml) and streptomycin (100 µg/ml). Cell lines were maintained in a 37°C humidified atmosphere with 5% CO_2. Cells were passaged every 2-3 days at approximately 80% confluence using 1% (w/v) Trypsin/EDTA solution in PBS. For collection of CM containing M-CSF, cells were seeded at $5x10^5$ cells/ml (40 ml) in a $T175^2$ culture flask and cultured for 7 days. CM was collected by centrifugation at 600 g for 6 min and supernatant collected. This centrifugation and supernatant collection step was repeated to ensure cell free CM. The supernatant was aliquoted and stored at -80°C until required. Cells were cultured for up to 25 passages before being discarded to prevent genetic instability accumulation.

2.2.1.9 Culturing of J558 Cells and GM-CSF Media Production

J558 cells were cultured in RPMI supplemented with 10% (v/v) heat inactivated FBS, penicillin G (100 µg/ml) and streptomycin (100 µg/ml). Blasticidin (10 µg/ml) was used to select for GM-CSF producing cells. Cell lines were maintained in a 37°C humidified atmosphere with 5% CO_2. The suspension cells were passaged every 3

days at approximately 80% confluence by centrifugation at 600 g for 6 min. For GM-CSF CM production, cells were cultured for 2 passages without Blasticidin. Cells were then seeded at $1x10^6$ cells/ml (40 ml) in a $T75^2$ culture flask. At 80% confluence, CM was collected by centrifugation at 600 g for 6 min and supernatant collected. This centrifugation and supernatant collection step was repeated to ensure cell free CM. The supernatant was aliquoted and stored at -80°C until required. Cells were cultured for up to 25 passages before being discarded to prevent genetic instability accumulation.

2.2.2 Molecular Biological Methods

2.2.2.1 Genomic DNA Extraction

For mouse colony genotyping, three week old mice were ear punched and tissue placed in sterile tubes. For genotyping MEFs, the embryo head was placed into a sterile tube. 100 µl of DirectPCR™ Lysis Reagent containing Proteinase K (0.4 mg/ml) was added to each tissue sample and incubated at 55°C overnight. Crude DNA lysates were subjected to a Proteinase K inactivation step in which samples were incubated at 85°C for 45 min. This lysate was used as the template for genotyping PCR reactions. Ear punch DNA was used neat, while DNA from embryos was diluted 1:10 in DNase free water.

2.2.2.2 Isolation of RNA and cDNA Synthesis

2.2.2.2.1 Isolation of Total RNA

In order to prevent RNA degradation by RNases, all working surfaces and areas were treated with an RNase decontamination solution. In addition, all plasticware used was certified RNase free. Gloves and face mask were also worn to prevent self-introduced RNase contamination.

For mRNA analysis experiments using BMDM and BMDCs, cells were seeded at $1x10^6$ cells/ml (3 ml) in 6-well plates. MEFs were seeded at $5x10^5$ cells/ml (3ml) in 6

well plates. HEK293-TLR3 cells were initially seeded at $7.5x10^4$ cells/ml (3 ml) in 6 well plates and subjected to genetic manipulation prior to harvesting. Total RNA was extracted using 500 µl Trizol Reagent following manufacturer's instructions. Briefly, cells were washed and centrifuged at 6,500 g for 7 min at 4°C. 500 µl of Trizol was added and pellets resuspended and left at room temperature for 5 min. 175 µl of chloroform was added and mixtures vortexed into a uniform suspension for 15 seconds (sec). Phase separation of aqueous RNA was achieved by incubating the lysates for 10 min at room temperature followed by centrifugation at 12,000 g for 15 min at 4°C. The clear interphase was removed and transferred to a fresh tube and an equal amount of isopropanol was added. After 5 min room temperature incubation, samples were vortexed for 5 sec and centrifuged at 12,000 g for 15 min at 4°C. The isopropanol was removed from the resulting RNA precipitated pellet and washed in 900 µl of 75% (v/v) ethanol and vortexed briefly. Samples were centrifuged at 6,500 g for 5 min at 4°C. The ethanol was removed and samples spun down again at 6,500 g for 5 min at 4°C. Any remaining ethanol was removed and the pellets were allowed to air dry for 10 min. Depending on pellet size, the RNA was resuspended in 15-50 µl RNase free water and incubated at 65°C for 10 min. The concentration of RNA was determined by using a Nanodrop™ spectrophotometer measuring absorbance at wavelengths of 260 nm and 280 nm, where absorbance of 1 unit at 260 nm is ~40 µg/ml. Pure RNA preparations had an $OD_{260/280}$ ratio of between 1.8-2.0. RNA was stored at -80°C until use as template for reverse transcriptase PCR.

2.2.2.2.2 Synthesis of First Strand cDNA from mRNA

In order to generate full-length first-strand cDNA from total cellular RNA, 2 µg of RNA, diluted in nuclease free water (12 µl volume), was added to a nuclease free microcentrifuge PCR tube. 1 µl of random primers (0.5 µg) were added to each RNA sample and incubated at 70°C for 5 min to remove any secondary structure which could impede long cDNA strand synthesis. The mixture was then chilled on ice prior to addition of the following:

Bioscript reverse transcriptase (200U/µl)	0.5µl

dNTPs (10mM)	1μl
5 x Bioscript Reaction Buffer	4μl
Nuclease-Free Water	1.5μl

The mixture was incubated for 10 min at room temperature to allow primers to anneal to the RNA. The reaction was then incubated at 42°C for 45 min. The reaction was terminated by incubation at 85°C for 5 min. The resulting cDNA was stored at -20°C.

2.2.2.3 PCR analysis

2.2.2.3.1 PCR Amplification

PCR products were amplified using isolated genomic or complementary DNA as template and using specific primers to detect the sequence of interest as outlined in 2.1.6.1 and 2.1.6.2 using the following reaction mixture:

Template DNA	2 μl
GoTaq polymerase	0.15 μl
5 x Buffer	5 μl
$MgCl_2$	2 μl
Primers (100 pmol/μl)	0.25 μl of sense
	0.25 μl of anti-sense
dNTPs (10 mM)	1 μl
Nuclease free water	14.35

Each sample was subjected to an initial incubation at 95°C for 5 min, followed by 29 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. Samples were then incubated at 72°C for 10 min and stored at 4°C.

2.2.2.3.2 Agarose Gel Electrophoresis

Agarose gels were prepared by adding 1.5% (w/v) agarose in TAE and heating until completely dissolved. The solution was then cooled to below 50°C and Ethidium Bromide (5 µg/ml) was added and solution poured into a gel tray. Following solidification, agarose gels were submerged in TAE buffer and subjected to electrophoresis at 90 volts. Samples were run simultaneously with a 1 kb molecular size marker. Nucleic acid products were visualised under ultraviolet (UV) light (254 nm) and images acquired using a Syngene G box gel documentation system.

2.2.2.3.3 Real-Time PCR

cDNA generated as described in 2.2.2.2.2 (diluted 1:10 with nuclease free water) was used for quantitative real-time PCR analysis using primes specific from gene sequences of interest as outline in 2.1.6.1. A reaction mix was made for each transcript containing:

2x SensiMix No Rox enzyme	10 µl
Primers (4 pmol/µl)	2.5 µl of sense
	2.5 µl of anti-sense
Nuclease free water	3 µl

18 µl of reaction mix was added to specialised optical 96 well plates and 2 µl template DNA added. Each sample was subjected to an initial 95°C for 15 min, followed by 45 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 45 sec. Intercalation of SYBR green into the dsDNA product formed was monitored after each step of primer annealing and elongation. Amplification of one specific product was confirmed by melt curve analysis whereby one single melting peak eliminated the possibility of primer-dimer associations. For melting curve analysis to be performed the products were heated from 60°C to 95°C after the 40 cycles. The PCR were conducted in a Applied Biosystems Step One™ real-time PCR instrument. The

relative quantification of target genes expression was evaluated using the ΔCT method. Crossing Threshold (CT) which recorded the cycle when sample fluorescence exceeds a chosen threshold above background fluorescence, was generated by Applied Biosystems Step One™ software. The ΔCT value was determined by subtracting the HPRT CT value for each sample from the target CT value. Fold change in the relative gene expression of target were determined by calculating the $2^{-\Delta CT}$.

2.2.2.4 Transformation of *E.coli* with Plasmid DNA

TOP10 chemically competent *E.coli* was used for propagation of plasmids. 100-400 ng (1 µl) of plasmid was added to 5 µl of TOP10 cells. DNA and the cells were mixed gently with a pipette and incubated on ice for 30 min. The plasmids were allowed to enter the bacterial cells by heat shocking the mixture at 42ºC for 40 sec. The cells become permeable to allow easy entry of the plasmid and cooling on ice for 2 min allowed the cells to once again become impermeable. The transformed cells were then incubated in 150 µl SOC medium at 37ºC on an Exceller E25 shaker at 220 revolutions per minute (rpm) for 1 hour (h). The mix was then plated out on LB agar plates (LB broth with 1.5% (w/v) agar) containing 100 µg/ml ampicillin. Plates were inverted and incubated overnight at 37ºC. Plates were then stored at 4ºC for up to four weeks.

2.2.2.5 Large-Scale Preparation of DNA from *E. coli*

100 ml LB broth containing ampicillin (50µg/ml) was inoculated with a single transformed *E. coli* colony from an agar plate. The culture was incubated overnight at 37ºC with constant shaking at 220 rpm. Large plasmid preparations were made using the high-speed plasmid midi kit from Qiagen. The bacterial cells were centrifuged at 3,000 g for 25 min. The supernatant was discarded and the plasmid DNA was extracted as outlined in the manufacturer's handbook. DNA concentration was determined by using a Nanodrop™ spectrophotometer measuring absorbance at wavelengths of 260 nm and 280 nm, where absorbance of 1 unit at 260 nm is ~50 µg/ml.

2.2.3 Genetic Manipulation of Mammalian Cells

2.2.3.1 Transient Transfection of MEFs Cells

MEFs derived from *Traf6*$^{-/-}$ mice were seeded at 5x10^{5} cells/ml (3 ml) in 6-well plates and left overnight until approximately 70% confluency. 1 ml of media was removed from all wells to be transfected. 3 µg of plasmid DNA for non-protein coding empty vector DNA (EV), TRAF6 or TRAF6$^{(C70A)}$ was diluted in 250 µl of OptiMEM and mixed gently. In addition, 4 µl of Lipofectamine 2000 was diluted into 250 µl of OptiMEM. After 5 min incubation the solutions were combined together and incubated for 20 min. 500 µl of the DNA-Lipofectamine complexes were added to each well and left for 48 h before stimulation and harvesting.

2.2.3.2 Transient Transfection of HEKT-TLR3 Cells

HEK293-TLR3 cells were seeded at 2x10^{5} cells/ml (3 ml) in 6-well plates and left overnight until approximately 70% confluency. 1 ml of media was removed from all wells to be transfected. 1 µg of plasmid DNA as indicated was diluted in 250 µl of OptiMEM and mixed gently. In addition, 4 µl of Lipofectamine2000 was diluted into 250 µl of OptiMEM. After 5 min incubation, the solutions were combined together and incubated for 20 min. 500 µl of the DNA-Lipofectamine complexes were added to each well and left for 24 h before harvesting.

2.2.3.3 Transfection of Cells for Knockdown with shRNA

HEK293-TLR3 cells were seeded at 7.5x10^{4} cells/ml (3 ml) in 6-well plates and left overnight. 1 ml of media was removed from all wells to be transfected. 2 µg of plasmid DNA encoding shRNA targeting Pellino3 transcript was diluted in 250 µl of OptiMEM and mixed gently. A control shRNA was also used in the transfection. It was a non-targeting shRNA vector that can activate the RNA-induced silencing complex and the RNAi pathway, but does not target any human genes. In addition, 4 µl of Lipofectamine 2000 was diluted into 250 µl of OptiMEM. After 5 min incubation the solutions were combined together and incubated for 20 min. 500 µl of

the DNA-Lipofectamine complexes were added to each well and left for 48 h. Cells were then subjected to treatment and harvesting or reconstituted (as indicated) by transient transfection outlined in 2.2.3.2.

2.2.3.4 Transfection of Cells for Knockdown with siRNA

HEK293-TLR3 cells were seeded at 7.5×10^4 cells/ml (3 ml) in 6-well plates and left overnight. 1 ml of media was removed from all wells to be transfected. 20 nM of siRNA targeting TRAF6 transcript or GAPDH as a control was diluted in 250 µl of OptiMEM and mixed gently. In addition, 4 µl of Lipofectamine 2000 was diluted into 250 µl of OptiMEM. After 5 min incubation the solutions were combined together and incubated for 20 min. 500 µl of the DNA-Lipofectamine complexes were added to each well and left for 48 h. Cells were then subjected transient transfection outlined in 2.2.3.2.

2.2.3.5 Retroviral Infection Assay

2.2.3.5.1 Generation of Viral Particles

HEK293T cells were seeded at 2×10^5 and grown overnight to 70% confluence in a $T175^2$ culture flask (25 ml). Cells were co-transfected with 8.5 µg of MCSV-EV plasmid or MCSV-mPeli3 or MCSV-mPeli3RING and with 8.5 µg of packaging (φ) vector using Lipofectamine 2000 as per manufactures instructions. In order to remove the transfection reagent and enhance retroviral production, the media was removed after 24 h and replaced with media containing 30% (v/v) FBS. The cells were then left for another 24 h and media containing the retrovirus harvested and subjected to centrifugation at 600 g for 6 min. The cell pellet was discarded and the supernatant again centrifuged. This supernatant constituted the cell free retrovirus used to infect MEF cells.

2.2.3.5.2 Retroviral Infection of MEFs

MEFs were seeded at the low density of 1×10^5 cells/ml (25 ml) in a $T175^2$ and allowed to adhere to the culture vessel, as the retrovirus only infects dividing cells.

The media was removed and replaced with 12.5 ml of retrovirus containing media for 6 hours and a further 12.5 ml of DMEM was then added to the flask and incubated for 48 h prior to experimental use.

2.2.4 Biochemical Methods

2.2.4.1 Protein Extraction

In order to extract intracellular protein from adherent cells, 6-well plates were placed on ice and media removed. Cells were washed with 3 ml of ice cold PBS and removed. Cells were scraped in 1 ml ice cold PBS and added to a 1.5 ml tube. Samples were centrifuged at 6,500 g for 10 min and PBS removed. For semi-adherent cells, the media containing the suspension cells was collected in a 15 ml tube. Adherent cells were then scraped in 1 ml of ice cold PBS and added to the 15 ml tube. Cells were centrifuged at 6,500 g for 10 min and PBS removed. Cell pellets were resuspended in 1 ml PBS and centrifuged at 6,500 g for 10 min and PBS removed. Regardless of cell type, pellets were then re-suspended in 90 µl cell lysis buffer and left on ice for 30 min. Samples were kept under near constant agitation by gently pipetting throughout the lysis process. Protein lysates were then subjected to centrifugation at 12,000 g for 10 min. 80 µl of the supernatant which constitutes the soluble intracellular protein was added to a new 1.5ml tube and stored at -20°C. Prior to loading proteins lysates, samples were mixed with 4x sample buffer and boiled for 5 min.

2.2.4.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the method of Laemmli as modified by Studier (Laemmli, 1970, Studier, 1973). Samples and appropriate prestained (10-180kDa) protein markers were loaded into separate wells. Electrophoresis was performed at 60V through a 5% polyacrylamide stacking gel and then through a 8-15%

polyacrylamide resolving gel at 80V for 1.5~2 h. The percentage gel was chosen based on the size of the proteins being electrophoresed.

2.2.4.3 Immunoblotting

Following separation by electrophoresis, the proteins were transferred electrophoretically to nitrocellulose membranes in a Hoefer TE 70 Semiphor semi-dry transfer unit at 110mA for 1.5 h. 3 layers of Whatmann paper were placed on the bottom surface of the transfer unit followed by one layer of nitrocellulose. The resolving gel was then placed on top with care to avoid any air bubbles. Finally 3 more layers of Whatmann paper were added and the unit closed. Following transfer, non-specific binding of antibody was blocked by incubating the nitrocellulose membranes at room temperature for 1 h with Blocking Buffer under gentle agitation. The membranes were then incubated under agitation at 4ºC overnight with the primary antibodies diluted in TBST containing 5% (w/v) skimmed milk powder or BSA as indicated in 2.1.3.1. The membranes were subsequently subjected to 3 x 5 min washes in TBST. Membranes were then incubated in a secondary antibody specific for the primary antibody in question (anti-rabbit or anti-mouse) in TBST containing 5% (w/v) skimmed milk powder for 1 h in the dark at room temperature. The membranes were then washed a further 3 times for 5 min each in TBST in the dark. The immunoreactive bands were detected using Odyssey infrared imaging system from Licor Biosciences or using enhanced chemiluminescence development.

2.2.4.4 Immunoprecipitation Analyses

2.2.4.4.1 Co-Immunoprecipitation of Proteins

For overexpression co-immunoprecipitation assays, cells were transfected with expression constructs as described in 2.2.3.2. 24 h post transfection, cell were placed on ice and media removed. For endogenous co-immunoprecipitation assays, cells were seeded at $1x10^6$ cells/ml (10 ml) in petri dishes and left overnight prior to stimulation (as indicated). Cells were washed in 3 ml ice cold PBS and scraped in 1 ml PBS and added to 1.5 ml tubes. Samples were centrifuged at 6,500 g for 10 min and PBS removed. Cell pellets were then re-suspended in 300 µl CoIP lysis buffer

and left on ice for 30 min. Samples were kept under near constant agitation by gently pipetting throughout the lysis process. Protein lysates were then subjected to centrifugation at 12,000 g for 10 min. 20 µl of this extract was removed and stored at -20°C and constituted whole cell lysate for loading and expression controls. For overexpression studies the remaining protein lysate was split into 2 tubes and the volume brought up to 1 ml in CoIP lysis buffer. Each tube was subjected to reciprocal co-immunoprecipitations using 1 µg of either anti-FLAG or anti-Myc antibody overnight at 4°C on a sample rocker. For endogenous interaction studies, the remaining protein lysate was immunoprecipitated with 1 µg of the antibody of interest overnight at 4°C on a sample rocker. Then 40 µl of protein A/G beads were added to each sample using pipettes with the top of each tip removed and samples were again left at 4°C overnight under constant agitation. Samples were centrifuged at 12,000 g for 1 min and supernatant removed. The pellets were washed with 1 ml CoIP lysis buffer and inverted several times. The samples were subjected to 3 more wash steps. Finally, all supernatant was removed from the pellet and 40 µl of 2x sample buffer was added to the beads and incubated at room temperature for 25 min. Samples were then boiled for 10 min and subjected to a brief vortex. Samples were then centrifuged at 12,000 g for 2 min and subjected to SDS-page as described in 2.2.3.2 and immunoblotting as described in 2.2.3.3.

2.2.4.4.2 Immunoprecipitation of Proteins to Investigate Post Translational Modifications

For overexpression studies investigating post translational modifications (PTM) of proteins, cells were transfected with expression constructs as described in 2.2.3.2. 24 h post transfection, cell were placed on ice and media removed. For investigation into stimulus induced PTMs of endogenous proteins, cells were seeded at $1x10^6$ cells/ml (10 ml) in a cell culture petri dish and left overnight prior to stimulation. In some experiments cells were initially subjected to RNAi as described in 2.2.3.3. In rescue experiments cells were infected with retroviral constructs as described in 2.2.3.5. Cells were stimulated as indicated and then placed on ice. For adherent cells, media was removed and washed in 3 ml ice cold PBS and scrapped in 1 ml PBS and added to 1.5 ml tubes. Samples were centrifuged at 6,500 g for 10 min and PBS

removed. For semi adherent cells, the media containing the suspension cells was collected in a 15 ml tube and the adherent cells were then scraped in 1 ml of ice cold PBS and added to the 15 ml tube. Cells were centrifuged at 4,200 g for 10 min and PBS removed. Cell pellets were re-suspended in 1 ml PBS and centrifuged at 6,500 g for 10 min and PBS removed. Cell pellets were then re-suspended in 200 µl IP lysis buffer and left on ice for 30 min. Samples were kept under near constant agitation by gently pipetting throughout the lysis process. Protein lysates were then subjected to centrifugation at 12,000 g for 10 min. 20 µl of this extract was removed and stored at -20°C and constituted whole cell lysate for loading and expression controls. To the remaining ~180 µl of protein lysis, 20 µl of 10% (w/v) SDS was added and samples left at room temperature for 5-10 min. The sample was then split into separate tubes and 900 µl of IP lysis buffer was added to dilute the SDS to ~0.1% (w/v) and samples returned to ice. Each tube was subjected to immunoprecipitation using 1 µg of the antibody of interest overnight at 4°C on a sample rocker. Then 40 µl of protein A/G beads were added to each sample using pipettes with the top of each tip removed and samples were again left at 4°C overnight under constant agitation. Samples were centrifuged at 12,000 g for 1 min and supernatant removed. The pellets were washed with 1 ml IP lysis buffer and inverted several times. The samples were subjected to 3 more wash steps. Finally, all supernatant was removed from the pellet and 40 µl of 2x sample buffer was added to the beads and incubated at room temperature for 25 min. Samples were then boiled for 10 min and subjected to a brief vortex. Samples were then centrifuged at 12,000 g for 2 min and subjected to SDS-page as described in 2.2.3.1.2 and immunoblotting as described in 2.2.3.1.2.

2.2.4.4 Enzyme-Linked Immunosorbent Assay (ELISA)

2.2.4.4.1 TNF-α, IL-6 and IP-10 ELISA

Samples were collected and stored at -80ºC until ELISA analysis. 96-well NUNC “Maxisorb” plates were coated with 100 µl of goat anti-mouse TNF-α (0.8 µg/ml), rat anti-mouse IL-6 (2.0 µg/ml) or rabbit anti-mouse IP-10 (0.5 µg/ml) diluted in PBS and were incubated overnight at room temperature. Plates were washed three times with wash buffer (PBS with 0.05% (v/v) Tween-20) and dried by forcefully

inverting the plates on tissue paper. Plates were blocked for 2 h with reagent diluent (PBS containing 1% (w/v) filtered sterilised (0.2 μm) BSA). Plates were again washed three times and dried. Samples or standards (100 μl) diluted in reagent diluent (1:1) were added to each well. The concentrations of standards were between 0 and 2000 pg/ml. Plates were incubated with samples or standards at 4°C overnight. 100 μl of biotinylated goat anti-mouse TNF-α (200 ng/ml), anti-IL-6 (150 ng/ml) or rabbit anti-mouse IP-10 (0.25 μg/ml) diluted in reagent diluent was added to each well. Plates were incubated for 2 h and washed as before. Streptavidin-HRP conjugate (100 μl) diluted 1:200 in reagent diluent was added to each well. The plates were incubated in the dark for 20 min and then the wash step was repeated. 100 μl TMB (1.25 mM/l) solution was added to each well and again plates were incubated in the dark for no longer than 20 min. 50 μl 1 N H_2SO_4 was used to stop the reaction and the OD was measured for each well at 450 nm and 590 nm using a ELx800TM microplate reader with Gen5 Data Analysis Software. The concentrations of TNF-α, IL-6 and IP-10 in each sample were extrapolated from a standard curve that related the OD of each standard to the known concentration. Standard samples were assayed in duplicate to generate the standard curve, while all samples were assayed in triplicate. Data analysis was performed using Graphpad Prism5 software.

2.2.4.4.2 IFN-β ELISA

Samples were collected and stored at -80ºC until ELISA analysis. 96-well NUNC "Maxisorb" plates were coated with 100 μl of rat anti-mouse IFN-β (0.1 μg/ml) diluted in PBS and were incubated overnight at room temperature. Plates were washed three times with wash buffer (PBS with 0.05% (v/v) Tween-20) and dried by forcefully inverting the plates on tissue paper. Plates were blocked for 2 h with reagent diluent (PBS containing 1% (w/v) filtered sterilised (0.2 μm) BSA). Plates were again washed three times and dried. Samples or standards (100 μl) diluted in reagent diluent (1:1) were added to each well. The concentrations of standards were between 0 and 5000 pg/ml. Plates were incubated with samples or standards at 4°C

overnight. 100 µl of unlabelled rabbit anti-mouse IFN-β (500 ng/ml) diluted in reagent diluent was added to each well. Plates were incubated for 2 h and washed as before. Anti-rabbit HRP-conjugate (100 µl) diluted 1:2000 in reagent diluent was added to each well. The plates were incubated for 1 h and then the wash step was repeated. 100 µl TMB (1.25 mM/l) solution was added to each well and again plates were incubated in the dark for no longer than 20 min. 50 µl 1 N H_2SO_4 was used to stop the reaction and the OD was measured for each well at 450nm and 590nm using a ELx800TM microplate reader with Gen5 Data Analysis Software. The concentrations of IFN-β in each sample were extrapolated from a standard curve that related the OD of each standard amount to the known concentration. Standard samples were assayed in duplicate to generate the standard curve, while all samples were assayed in triplicate. Data analysis was performed using Graph pad Prism5 software.

2.2.4.4.3 IFN-α ELISA

Samples were collected and stored at -80ºC until ELISA analysis which was conducted using the IFN-α Platinum ELISA Kit from eBiosciences. Microwell plate strips coated with monoclonal antibody against murine IFN-α2/4 and were washed twice with the wash buffer (300 µl) provided and dried on tissue. 50 µl of Assay buffer was added to all wells. 50 µl of standards ranging from 31.3 pg/ml to 2000 pg/ml was added to designated wells. 50 µl of calibration buffer was added to act as a blank (0 pg/ml). 50 µl of supernatant was added to each sample well. Finally 50 µl of Biotin-Conjugate solution was added to all wells and the microwell plate sealed with an adhesive film and incubated for 2 h at room temperature at 400 RPM. After incubation, the plate was washed 4 times and 100 µl of the Streptavidin-HRP solution was added to each well and the plate re-sealed with a new adhesive strip. The plate was incubated at room temperature for 1 h at 400 RPM. The plate was then washed another 4 times and 100 µl of the substrate TMB solution was added to each well and incubates in the dark at room temperature for 30 min. The reaction was stopped by the addition of 100 µl Stop Solution. The OD was measured for each well at 450nm and 590nm using a ELx800TM microplate reader with Gen5 Data Analysis Software. The concentrations of IFN-β in each sample were extrapolated

from a standard curve that related the OD of each standard amount to the known concentration. Standard samples were assayed in duplicate to generate the standard curve, while all samples were assayed in triplicate. Data analysis was performed using Graph pad Prism5 software.

2.2.4.5 Electro Mobility Shift Assay (EMSA)

EMSA was used to detect the presence of DNA-binding proteins in nuclear extracts. NF-κB and PRDI/III Infrared Dye labelled oligonucleotides was purchased from Licor Biosciences and MWG respectively.

2.2.4.5.1 Preparation of Sub-Cellular Fractions

MEF cells were seeded at $5x10^5$ cells/ml (3 ml) or BMDCs were seeded at $1x10^6$ cells/ml (3 ml) for generation of subcellular reactions. Following treatment, cells were washed with 1 ml of ice cold PBS and then scraped into 1 ml of hypotonic Buffer A. Following centrifugation at 4ºC at 16,000 RPM for 10 min the supernatants were removed and the cells were lysed for 10 min on ice in Buffer A containing 0.1% (w/v) Igepal (20 µl). Lysates were centrifuged at 21,000 g for 2 min. The resulting supernatants constituted cytosolic extracts and were stored at -20ºC in fresh 1.5 ml tubes. The remaining pellets were resuspended in 20 µl of Buffer B and incubated on ice for 20 min and kept under constant agitation. Samples were then centrifuged at 16,000 RPM for 10 min. The supernatants were then transferred to 50 µl of Buffer C and were considered as the nuclear extracts. Protein concentrations were determined by the method of Bradford and assayed for translocation by western blotting (as described in 2.2.3.1 – 2.2.3.3) or assayed for DNA-binding activity as outlined in the following sections.

2.2.4.5.2 Bradford Assay

In order to determine the concentration of protein in nuclear extracts a Bradford assay was conducted as previously described (Bradford, 1976). BSA standards of a known protein concentration and samples were diluted in water (20 µl) and mixed with 180 µl of Bradford assay reagent and protein concentration determined by the

formation of a colorimetric product. The OD was measured for each well at 590nm using a ELx800TM microplate reader with Gen5 Data Analysis Software. The concentrations of protein in each sample were extrapolated from a standard curve that related the OD of each standard amount to the known concentration. Standard samples were assayed in duplicate to generate the standard curve, while all samples were assayed in triplicate. Data analysis was performed using Graph pad Prism5 software.

2.2.4.5.3 EMSA Reaction

Nuclear extracts (5µg of protein), generated as described in section 2.2.3.4.1, were incubated with the following reaction mix:

10x Binding Buffer	2 µl
Poly (dI-dC)	1 µl
25mM DTT	2 µl
1% NP40	1 µl
1mM $MgCl_2$	1 µl
IRDye labelled / non-labelled oligonucleotides	1 µl
Nuclear extract (5 µg) + H_20	12 µl

NF-κB consensus oligonucleotide:

5’ - AGTTGAGGGGACTTTCCCAGGC - 3’

PRDI/III consensus oligonucleotide:

5’ - GTAAATGACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGG - 3’

For competition assays, unlabelled oligonucleotides containing the consensus NF-κB were added for 10 min at room temperature, prior to addition of the IRDye labelled oligonucleotides. Incubations were performed in the dark for 30 min at room temperature. Reactions were stopped by adding 10X orange loading dye and placing reactions on ice prior to loading onto an native gel.

2.2.4.5.4 Native Acrylamide Gel Electrophoreisis

A native 5% Acrylamide Gel was prepared as follows

H_2O	39ml
10x TBE	2.5ml
30% Polyacrylamide	8.4ml
10% APS	250µl
TEMED	20µl

The components were mixed well while minimizing bubble formation. The gel was poured and allowed polymerize for 30-45 min. The gel was pre-run in 0.5X TBE for 30 min at 70V. The samples were added and subjected in the dark to electrophoresis for ~90 min at 70V in the non-denaturing 5% (w/v) polyacrylamide gel. Gels were then removed from the glass plates and visualised using Odyssey Infrared Imaging System from Licor Biosciences.

Chapter 3: Results

Defining the Physiological Role of Pellino3 in Toll-like Receptor 3 Signalling

3.1 Prologue

The initiation of the innate immune response is paramount to pathogenic identification and host protection. A family of PRRs known as the TLRs play a fundamental role in this process by both recognising and responding to specific pathogenic insults. Cell surface TLR1/2/4/5/6 have been shown to bind a vast array of microbial PAMPs ranging from gram positive and negative bacteria to fungal and parasitic motifs (McGettrick and O'Neill, 2010). In contrast, TLR7/8/9 are located on intracellular endosomes whereby they detect and respond to bacterial and viral nucleic acids (Blasius and Beutler, 2010). Interestingly, TLR3 a critical mediator of anti-viral defence against dsRNA species, is located on both endosome and cell surface membranes in a temporal and cell specific manner (Seya *et al*., 2009). Upon TLR-PAMP ligation, receptor dimerisation allows recruitment of the TIR adaptor proteins to instigate complex downstream signalling cascades which can be broadly categorised into the Myd88-dependent and TRIF-dependant pathways. Both pathways culminate in the transactivation of key transcription factor families, such as NF-κB, AP-1 and the IRFs leading to the co-ordinated induction of key mediators of host defence (Medzhitov *et al*., 1997b, Kyriakis *et al*., 1994, Medzhitov *et al*., 1998a, Sato *et al*., 2000).

TLR3 is unique in the TLR family in that it alone does not require Myd88 to propagate downstream signalling (Oshiumi *et al*., 2003a). TLR3 exclusively recruits TRIF to its cytosolic TIR domain upon viral dsRNA ligation leading to TRIF phosphorylation by Burtons tyrosine kinase (BTK) (Lee *et al*., 2012). Phosphorylated TRIF can activate the NF-κB pathway in two distinct ways; a TRAF6-mediated pathway and a RIP1-dependent pathway. It has been reported that TRAF6 can associate with the N-terminus of activated TRIF, leading to TRAF6 oligomersiation and activation of its E3 ubiquitin ligase activity (Sato *et al*., 2003, Ea *et al*., 2004). Polyubiquitinated TRAF6 on Lysine124 acts a signalling platform for the recruitment of a TAK1/TAB1/TAB2/TAB3 complex allowing TAK1 to become autophosphorylated (Jiang *et al*., 2003b, Kishimoto *et al*., 2000, Lamothe *et al*., 2007a). The formation of polyubiquitin chains by TRAF6's E3 ligase domain

also serves to bring TAK1, a MAPKKK, into close proximity to its substrates IKKs and MKKs (Wang *et al*., 2001, Ea *et al*., 2004, Lamothe *et al*., 2007b). TAK1 promotes phosphorylation of the IKKs which in turn localise to and phosphorylate IκBα (Zandi *et al*., 1998). Phosphorylation of IκBα leads to its K48-linked polyubiquitination and proteasomal degradation allowing NF-κB to translocate to the nucleus and initiate gene transcription (Brown *et al*., 1995). Recently however, a number of studies have reported contrasting roles for TRAF6 in TLR3 dependent NF-κB activation. TLR3-induced activation of NF-κB is completely abolished in TRAF6-deficient MEFs. However, TRAF6-deficient macrophages display normal NF-κB activity (Jiang *et al*., 2003b, Gohda *et al*., 2004). These discrepancies may be explained in part by cell type specific roles for TRAF6 and/or by functional redundancy with other members of the TRAF family, such as TRAF2 (Sasai *et al*., 2010) . In addition, the role of TRAF6 in bridging TRIF to the NF-κB pathway may be mediated in full by the RIP1-dependent mechanism. Upon TLR3 dimerisation, RIP1 is recruited to TRIF's C-terminus via a RIP homotypic interaction motif (Meylan *et al*., 2004). The adaptor protein TRADD binds RIP1 and is required for the recruitment of an E3 ligase to facilitate RIP1 polyubiquitination and subsequent interaction with the TAK1 complex to propagate NF-κB activation (Ermolaeva *et al*., 2008, Cusson-Hermance *et al*., 2005). Although TRAF6 was originally proposed as the E3 ligase initiating RIP1 polyubiquitination, due to its cell type specific roles, other E3 ligases must be critical for this modification. Recently a member of the Pellino family of E3 ubiquitin ligases Pellino1 was shown to be vital for TRIF-induced activation of NF-κB and pro-inflammatory cytokine production. Pellino1 interacts with RIP1 and induces TRIF- dependent RIP1 polyubiquitination and NF-κB pathway activation (Chang *et al*., 2009). As Pellino1 is required for NF-κB activation in both macrophages and MEFs, it indicates that the Pellino protein may be the key E3 ligase for TRIF dependent NF-κB signalling.

In addition to inducing NF-κB and MAPK activity, TRIF-dependent signalling can also trigger the activation of the interferon regulatory factors IRF3 and IRF7. In TLR3 signalling the activation of IRF3 has been intensively investigated, with studies on IRF7 mainly restricted to TLR7/8/9 signalling. The TRIF dependent activation of IRF3 activation is independent of TRAF6 and RIP1 (Cusson-Hermance

et al., 2005, Jiang *et al*., 2004). Instead, TRIF recruits both the adaptor protein NAP1 and the E3 ligase TRAF3 to propagate downstream activation of the non-canonical IKKs, TBK1 and IKKi (Sasai *et al*., 2005, Hacker *et al*., 2006, Oganesyan *et al*., 2006). NAP1 is thought to directly facilitate the interaction of TBK1 and IKKi with the N-terminus of TRIF while K63-linked ubiquitination of TRAF3 has been proposed as a requirement for their downstream kinase activity. Activated TBK1 and IKKi can in turn bind to and phosphorylate IRF3/7 leading to their dimerisation, nuclear translocation and upregulation of anti-viral cytokines (Sharma et al., 2003, McWhirter et al., 2004b, Hiscott et al., 1999, Hemmi et al., 2004). IRF7 activation has been extensively investigated in specialised anti-viral response cells known as pDCs (Gilliet *et al*., 2008). Interestingly, pDCs sense viral nucleic acids by TLR7/9 (Kadowaki *et al*., 2001, Kato *et al*., 2005). pDC activation of TLR7/9 results in the specific recruitment of the TIR adaptor protein Myd88 (Hemmi et al., 2003a). This causes MyD88 to complex with TRAF6 and IRF7 allowing TRAF6 to activate IRF7 via K63-linked ubiquitination followed by nuclear translocation and type 1 IFN production (Kawai *et al*., 2004). Therefore, although TRAF6's role in NF-κB activation during anti-viral signalling is controversial its ability to activate IRF7, at least in pDCs, is undisputed.

Type 1 IFN upregualtion by TLR3 is critical for host protection to a number of viral infections and requires the co-ordinated activation of NF-κB, AP-1 and IRF3/7 at the promoter of responsive genes. The best characterised example is the enhanceosome of the IFN-β promoter and contains at least four regulatory cis-elements: the positive regulatory domains (PRDs) I, II, III and IV. It is known that NF- κB binds to the PRDII element, AP-1 binds to PRDIV while IRF3 and IRF7 bind to the PRDI/III regions of the promoter (Du and Maniatis, 1992, Tanaka *et al*., 1993, Fujita *et al*., 1989). Interestingly the PRDII and PRDIV domains have yet to be described for the IFN-α promoter partly explaining how IRF7 alone is a major driver of this cytokine by pDCs (Honda *et al*., 2005b). IFN-α exists as a family of structurally related cytokines while IFN-β is a single protein subtype (Bekisz *et al*., 2004). Interestingly, it is thought that IFN-α is functionally active as a monomer while the IFN-β functional unit is a dimer, however the biological significance of this is as yet unknown (Kempner and Pestka, 1986, Pestka *et al*., 1983, Pestka *et al*., 2004).

During initial TLR3 signalling both IFN-α4 and IFN-β are strongly upregulated and secreted from the cell and signal in an autocrine and paracrine fashion. This signalling is mediated by the type 1 IFNα/β receptor (IFNAR) which consists of two subunits known as IFN-αR1 and IFN-αR2c (Uze *et al.*, 1990, Novick *et al.*, 1994). IFN-αR1 is basally associated with tyrosine kinase 2 (Tyk2) while the IFN-αR2c is thought to interact with Janus kinase 1 (Jak1) (Domanski *et al.*, 1997, Yan *et al.*, 1996). Ligand-induced receptor rearrangement and subunit dimerisation initiate tyrosine phosphorylation and activation of the associated kinases (Silvennoinen *et al.*, 1993, Kotenko *et al.*, 2003). This in turn leads to tyrosine phosphorylation of a number of signal transducer and activator of transcription (STAT) proteins, including STAT1 and STAT3 (Matikainen *et al.*, 1999). The activated STATs then form heterodimers or homodimers and translocate to the nucleus where they initiate transcription of IFN-stimulatory genes (ISGs) (Darnell, 1997). The formation of ISG factor 3 (ISGF3) complex includes activated STAT heterodimers and IRF9 allows for the induction of genes containing an IFN-stimulated response element (ISGE) (Fu *et al.*, 1990, Schindler *et al.*, 1992). Other combinations of STAT oligomers can also bind another response element known as the IFN-γ-activated site (GAS) element, that is present in the promoter of many ISGs (Ghislain *et al.*, 2001). Type 1 IFN upregulation and activation of PKR leads to phosphorylation and termination of viral translation by initiation factor eIF2α upon dsRNA viral detection (Meurs *et al.*, 1990, Balachandran *et al.*, 2000). In addition, upregulation of 2',5'-oligoadenylate synthetase (OAS) and RNase L lead to direct degradation of viral mRNA (Zhou *et al.*, 1993, Zheng *et al.*, 2001). Upregualtion of chemokines such as interferon γ-induced protein 10 (IP-10) can result in the recruitment of cytotoxic $CD8^+$ T cells and cell death in virus infected cells represent another mechanism ISGs use to protect host homeostasis (Klein *et al.*, 2005). As TLR activation of type 1 IFNs can have dramatic consequences to the host, it is therefore critical the initial induction is controlled.

It is clear that ubiquitination is an important mediator of TLR signal transduction. An emerging family of E3 ubiquitin ligases, the Pellino proteins, have recently been implicated in TLR signalling (Moynagh, 2009). The three mammalian Pellino proteins, Pellino1, Pellino2 and Pellino3 share an extremely high sequence

homology (Schauvliege *et al*., 2007). Each member of the Pellino family contains a N-terminal forkhead-associated (FHA) domain that recognises phosphothreonine residues and mediates association with IRAK proteins (Lin *et al*., 2008). In addition, each Pellino molecule contains a conserved C-terminal RING-like domain that confers the E3 ubiquitin ligase activity to the family (Schauvliege *et al*., 2006, Butler *et al*., 2007). Pellino1 is the only Pellino protein to be defined physiologically in TLR signalling using gene targeted studies. As mentioned above, Pellino1 is critically important for TRIF dependent activation of NF-κB during TLR3/4 signalling (Chang *et al*., 2009). Reports on the functionality of the other Pellino proteins have been reliant on RNAi and overexpression approaches and have provisionally linked them to MAPK activation (Jensen and Whitehead, 2003a, Jensen and Whitehead, 2003b, Butler et al., 2005). In this thesis, the generation of a *Peli3*$^{-/-}$ mouse is described and physiological function of Pellino3 in TLR3 signalling is defined.

3.2 Results

3.2.1 Design Strategy and Genotyping of a *Peli3*$^{-/-}$ Mice

To date, the function of Pellino3 in TLR signalling has remained elusive. Functional studies regarding Pellino3 have relied solely on overexpression and RNAi studies. In order to elucidate its physiological role in TLR immunobiology, Pellino3-deficient mice were generated according to the strategy outlined (Fig 3.1a). Using its proprietary technology, Taconic Artemis generated ES cells in which the *Peli3* gene was flanked with loxP sites. A targeting vector was designed using BAC clones from the C57BL/6J RPCI-23 BAC library and transfected into the Taconic Artemis C57BL/6N Tac ES cell line. The constructed vector flanked exon3 of the *Peli3* gene with the addition of a positive selection marker inserted into intron2. Generation of homologous recombinant clones were isolated using positive (Puromycin resistance) and negative (Thymidine kinase) selections and used to create chimeric mice by blastocyst injection. Highly chimeric males were chosen and mated with C57BL/6 females. Their heterozygous progeny were identified by Southern blotting. Heterozygous mice with the targeted exon3 were bred with mice expressing Cre recombinase regulated by the ROSA26 locus (C57BL/6-*Gt(ROSA)26Sor*$^{tm16(Cre)Arte}$). Upon recombination, exon3 of the *Peli3* gene was deleted and caused a downstream frameshift in exons4-7. The Cre transgene was subsequently eliminated by mating the generated *Peli3*$^{(+/-)}$ with WT C57BL/6 mice during colony expansion.

In order to determine the genotype of the mice used in this study, PCR analysis of DNA isolated from murine ear punches was conducted (Fig. 3.1b). Genotyping PCR using WT (*Peli3*$^{(+/+)}$) genomic DNA as a template resulted in the production of a large 1156 bp fragment when using primers (a + b) for the full length *Peli3* gene spanning exon3. Genotyped DNA from heterozygous (*Peli3*$^{(+/-)}$) animals also resulted in this large PCR product. However, the presence of a smaller 332 bp fragment detecting the recombined *Peli3* allele was also observed. This smaller fragment was the only amplified product when using the *Peli3*-deficient (*Peli3*$^{-/-}$) mice DNA as template. Primers (c + d) were used to distinguish heterozygous and

homozygous mice. Primer d was designed to target a part of the *Peli3* gene sequence which was inside the loxP flanking region before exon3. Upon recombination this site was deleted. Genotyping PCR using this primer set resulted in a 210 bp fragment from both WT and heterozygote genomic DNA, but not from the *Peli3*-deficient DNA confirming the genotype of both alleles. For all reactions, DNase free water (H_20) was used as a template as a negative control. As a positive control, primers were used to amplify the housekeeping gene *HPRT*.

3.2.2 Lack of Pellino3 Expression in *Peli3*$^{-/-}$ Murine Cells

Semi-quantitative RT-PCR (Fig. 3.2a) was conducted on mRNA from MEFs, BMDMs and BMDCs using primers specific for either exon3 of Pellino3 or HPRT. The RT-PCR from the WT mice generated a PCR product in all 3 cell types. This band was completely absent in the *Peli3*-deficient cells. For this reaction, DNase free water was used as a template as a negative control. In addition, quantitative real-time RT-PCR (Fig. 3.2b) was performed to measure the levels of Pellino3 mRNA in the cell types used in this thesis. The total mRNA levels of each cell type were equalised to expression of HPRT mRNA and Pellino3 expression presented as a percentage of the indicated WT cells. A total abrogation of Pellino3 gene expression was evident in all cell types in *Peli3*$^{-/-}$ mice samples. Taken together, these studies demonstrate that Pellino3 gene expression is absent in the *Peli3*$^{-/-}$ mice.

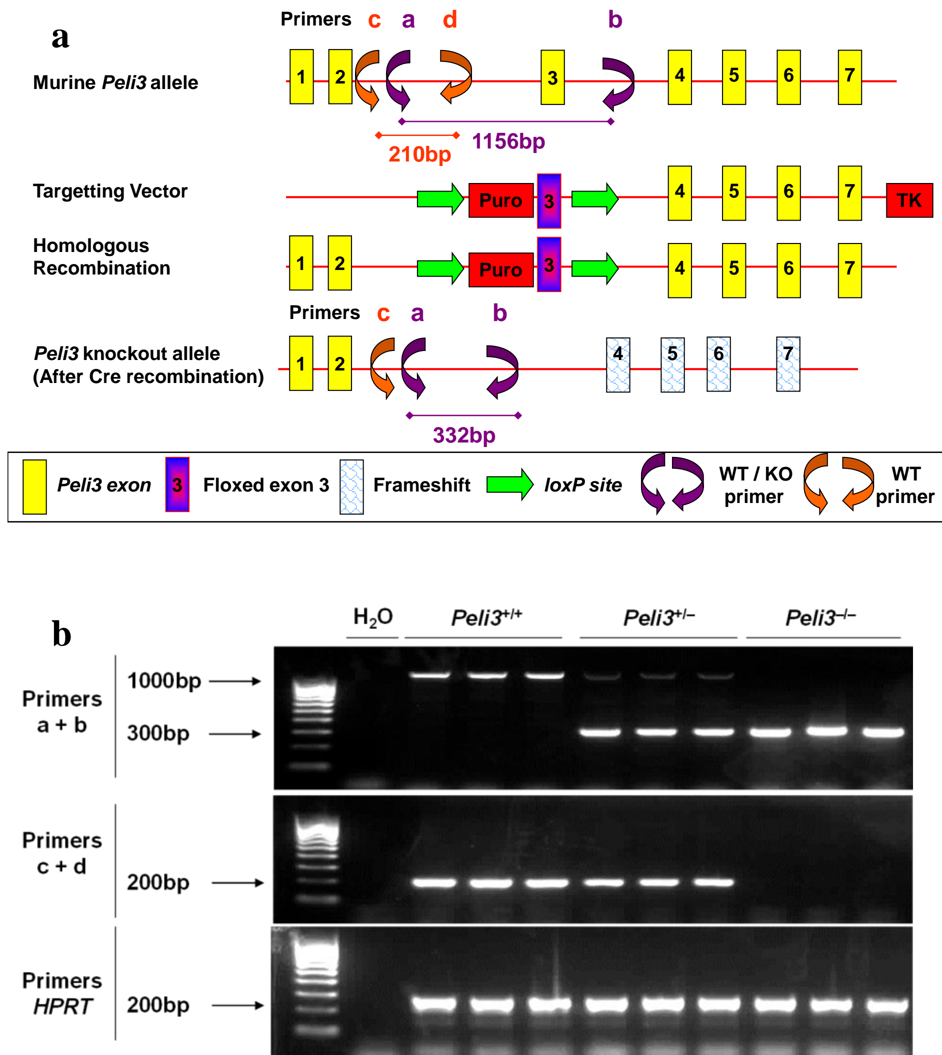


Figure 3.1 Deletion of the murine *Peli3* gene.

(a) Strategy used to generate *Peli3*-deficient mice. Diagram shows the murine *Peli3* gene; the *Peli3* targeting vector containing exon3 flanked by loxP sites, the positive selection marker (Puro) inserted into intron2 and a downstream Thymidine Kinase (TK) gene; the targeted *Peli3* allele; and the *Peli3* knockout allele after Cre recombination. Homologous recombinants in ES cells were isolated by Puro selection and used for blastocyst injection. Mice that were heterozygous for the targeted allele were bred with mice containing Cre recombinase regulated by the ROSA26 locus generating constitutive *Peli3*-deficient mice in which deletion of exon3 results in loss of function of the *Peli3* gene by generating a frameshift in all downstream exons. Exons are numbered and regions targeted by genotyping primers are also indicated. **(b)** Genotyping by PCR analysis of genomic DNA from ear punches. Primers a and b differentiate the WT allele from the *Peli3* knockout allele in heterozygous and homozygous mice based on different fragment size where primers c and d amplify a larger fragment for the WT allele and a shorter fragment for the knockout allele. Primers c and d detect only the WT *Peli3* allele. Primers for HPRT were used to assess equal genomic DNA content in all reactions. Water was used as a negative control for the PCR reaction.

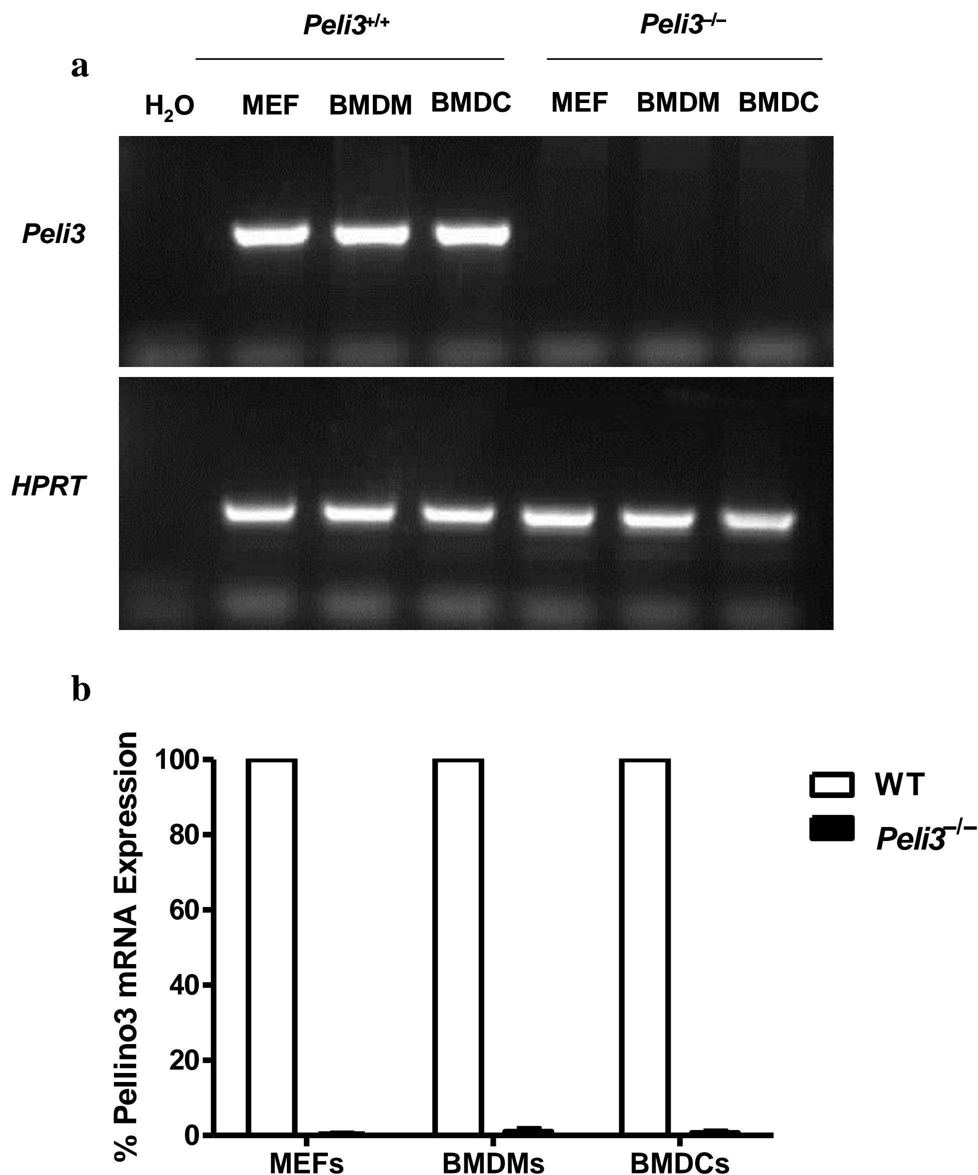


Figure 3.2 Expression of Pellino3 mRNA in cells isolated from WT and *Peli3*$^{-/-}$ mice.

Murine embryonic fibroblasts (MEF), bone marrow derived macrophages (BMDM) and bone marrow derived dendritic cells (BMDC) were isolated from WT and *Peli3*$^{-/-}$ mice. **(a)** cDNA was generated and semi-quantitative RT-PCR was performed using primers for exon3 of the *Peli3* gene or a region of the house-keeping gene HPRT. Water was used as a negative control. **(b)** Real time PCR was performed to quantify the expression levels of the Pellino3 mRNA in the indicated cell types. All mRNA levels were normalised to HPRT expression. Pellino3 levels in *Peli3*$^{-/-}$ cells are measured as a % of their WT counterparts.

3.2.3 Loss of Pellino3 Fails to Affect TLR-induced Expression of TNF-α

The role of Pellino3 in TLR signalling was initially explored by comparing the expression of TLR-responsive genes in cells from WT and Pellino3-deficient mice. BMDMs (Fig. 3.3a) and BMDCs (Fig. 3.3b) from WT and *Peli3*$^{-/-}$ mice were stimulated for 6 h with ligands for TLR2/6, Pam2CSK; TLR2/1, Pam3CSK; TLR2 and Dectin-1, Zymosan; TLR3, Poly(I:C); TLR4, LPS; TLR5, Flagellin; TLR7/8, CLO97; and TLR9, CpG type B and mRNA levels assessed for the pro-inflammatory cytokine TNF-α by quantitative real-time PCR. Each TLR agonist induced expression of TNF-α in all cell types from WT and *Peli3*$^{-/-}$ mice. However, no differences in cytokine upregulation were detected in cells deficient in Pellino3 compared to that of WT cells.

Although TNF-α mRNA expression was comparable between the WT and Pellino3-deficent cells after 6 h TLR stimulation (Fig. 3.3), it was important to confirm Pellino3 had no role in TNF-α protein production and secretion. To this end, BMDMs (Fig. 3.4a), BMDCs (Fig. 3.4b) and MEFs (Fig. 3.4c) were stimulated with the TLR ligands as indicated for 24 h. Again all ligands triggered TNF-α production in each cell type and no differences in TNF-α induction were observed between cells derived from WT and *Peli3*$^{-/-}$ mice. These results indicate Pellino3 is dispensable for TLR-induced expression of TNF-α.

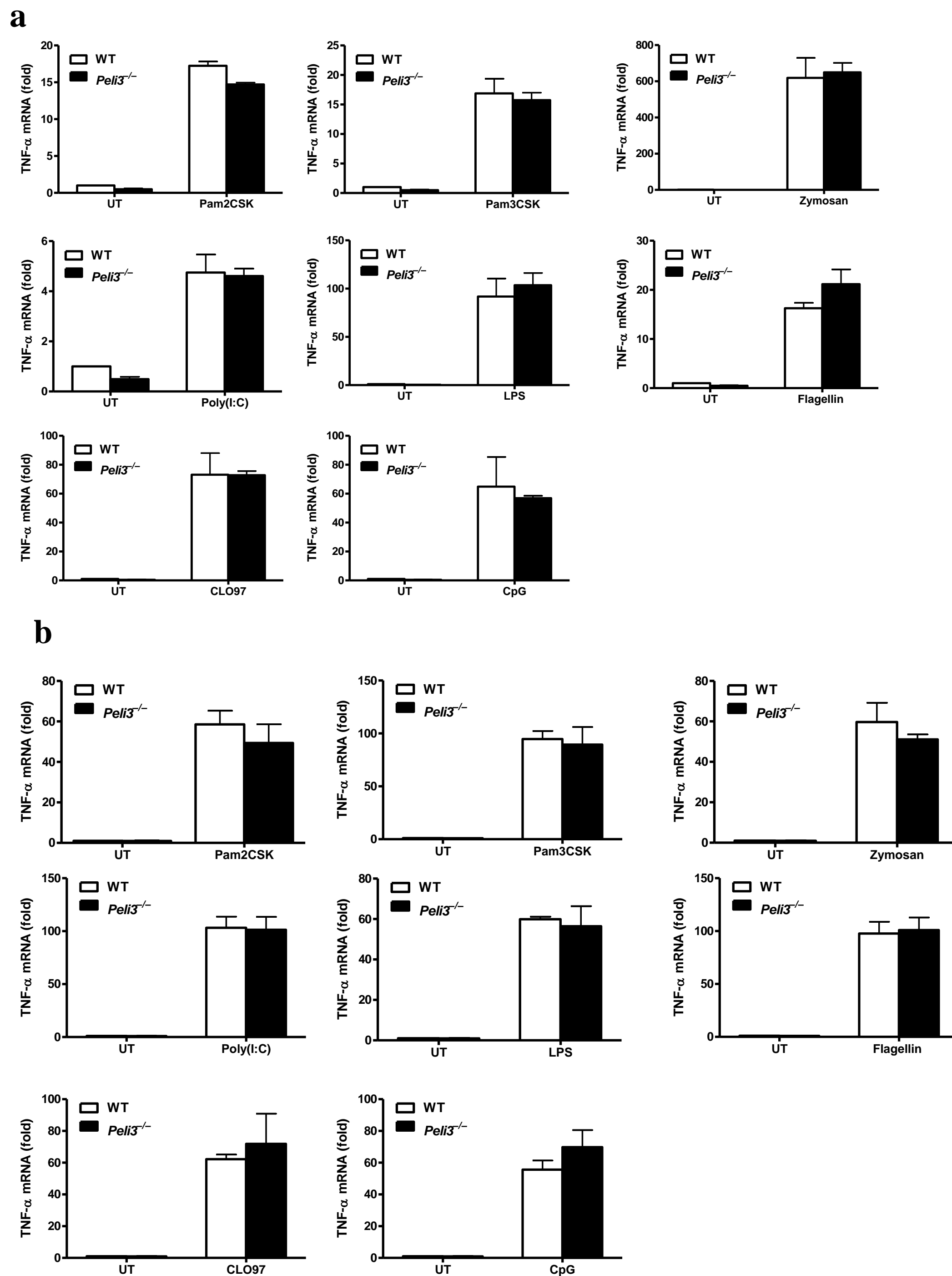


Figure 3.3 Effect of Pellino3-deficiency on TLR-induced expression of TNF-α mRNA.

Quantitative real-time PCR of TNF-α expression in cells from **(a)** BMDMs or **(b)** BMDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Pam_2CSK (10 ng/ml), Pam_3CSK (10 ng/ml), Zymosan (5 µg/ml), Poly(I:C) (10 µg/ml), LPS (10 ng/ml), Flagellin (1 µg/ml) or CLO97 (1 µg/ml) or CpG (3 µM) for 6 h. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t*-test.

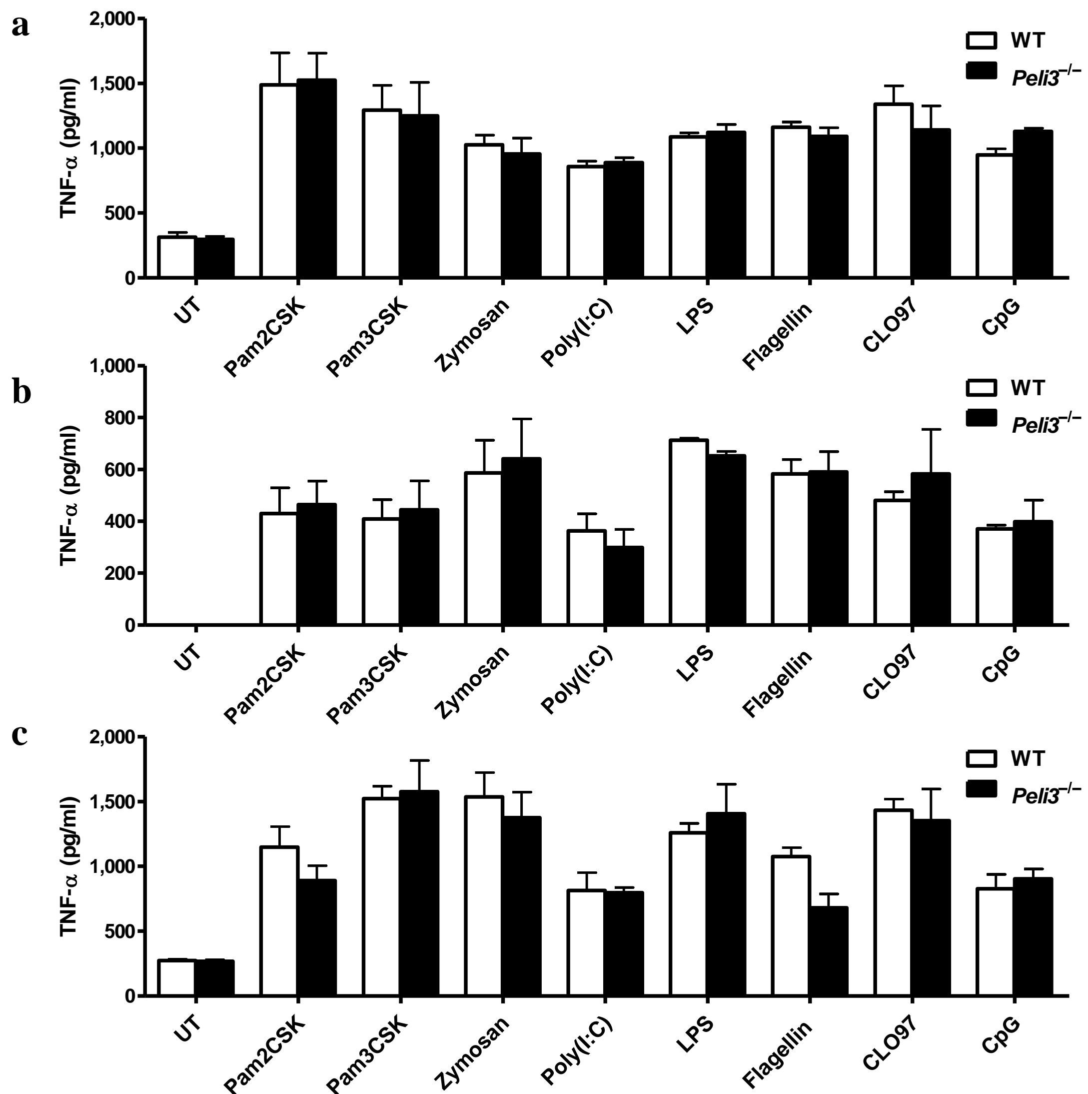


Figure 3.4 Effect of Pellino3-deficiency on TLR-induced production of TNF-α.

ELISA of TNF-α production in media from (**a**) BMDMs or (**b**) BMDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Pam_2CSK (10 ng/ml), Pam_3CSK (10 ng/ml), Zymosan (5 μg/ml), Poly(I:C) (10 μg/ml), LPS (10 ng/ml), Flagellin (1 μg/ml), CLO97 (1 μg/ml) or CpG (3 μM) for 24 h. ELISA of TNF-α production in media from (**c**) MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Pam_2CSK (100 ng/ml), Pam_3CSK (100 ng/ml), Zymosan (5 μg/ml), Poly(I:C) (25 μg/ml), LPS (100 ng/ml), Flagellin (5 μg/ml), CLO97 (5 μg/ml) or CpG (5 μM) for 24 h. Higher concentrations of the each TLR ligand was used in MEF cells as they are not as responsive to TLR activation as professional innate immune cells. Data are presented as the mean +/- S.E.M. of least three independent experiments and were subjected to paired two-tailed Student's *t* test.

3.2.4 Loss of Pellino3 Fails to Affect TLR-induced Expression of IL-6

A potential role for Pellino3 in TLR-induced expression of IL-6 was next examined with a view to assessing if the lack of an involvement of Pellino3 in TNF expression extended to other pro-inflammatory cytokines. BMDMs (Fig. 3.5a) and BMDCs (Fig. 3.5b) from WT and *Peli3*$^{-/-}$ were stimulated for 6 h with the indicated TLR agonists and levels of IL-6 mRNA expression assessed by quantitative real-time PCR. Each TLR ligand induced an upregulation of IL-6 expression in cells from WT and Pellino3-deficent animals. However, no significant differences in IL-6 mRNA were observed between WT and Pellino3-deficent cells.

In order to confirm Pellino3 had no role in TLR production of IL-6 protein, BMDMs (Fig. 3.6a), BMDCs (Fig. 3.6b) and MEFs (Fig. 3.6c) were stimulated with the TLR ligands as indicated for 24 h. TLR stimulation with all ligands resulted in increased Il-6 production in all cell types. Once again however, Pellino3-deficiency had no effect on the pro-inflammatory cytokines expression when compared to WT cells. Taken together these results indicate Pellino3 is dispensable during TLR induction of IL-6 and strongly suggests it has no role in pro-inflammatory cytokine expression during TLR signalling.

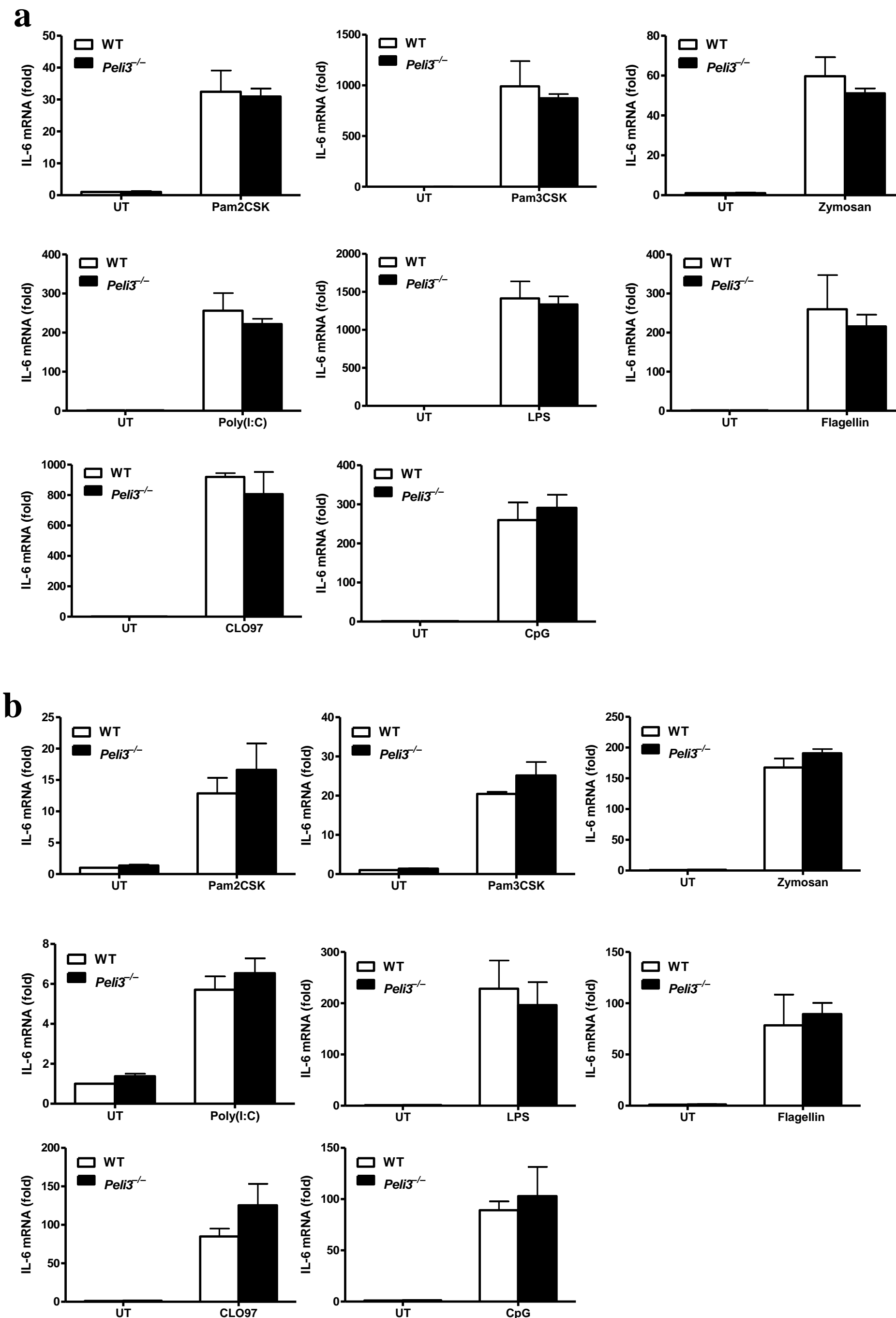


Figure 3.5 Effect of Pellino3-deficiency on TLR-induced expression of IL-6 mRNA.

Quantitative real-time PCR of TNF-α expression in cells from **(a)** BMDMs or **(b)** BMDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Pam$_2$CSK (10 ng/ml), Pam$_3$CSK (10 ng/ml), Zymosan (5 µg/ml), Poly(I:C) (10 µg/ml), LPS (10 ng/ml), Flagellin (1 µg/ml) or CLO97 (1 µg/ml) or CpG (3 µM) for 6 h. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's t-test.

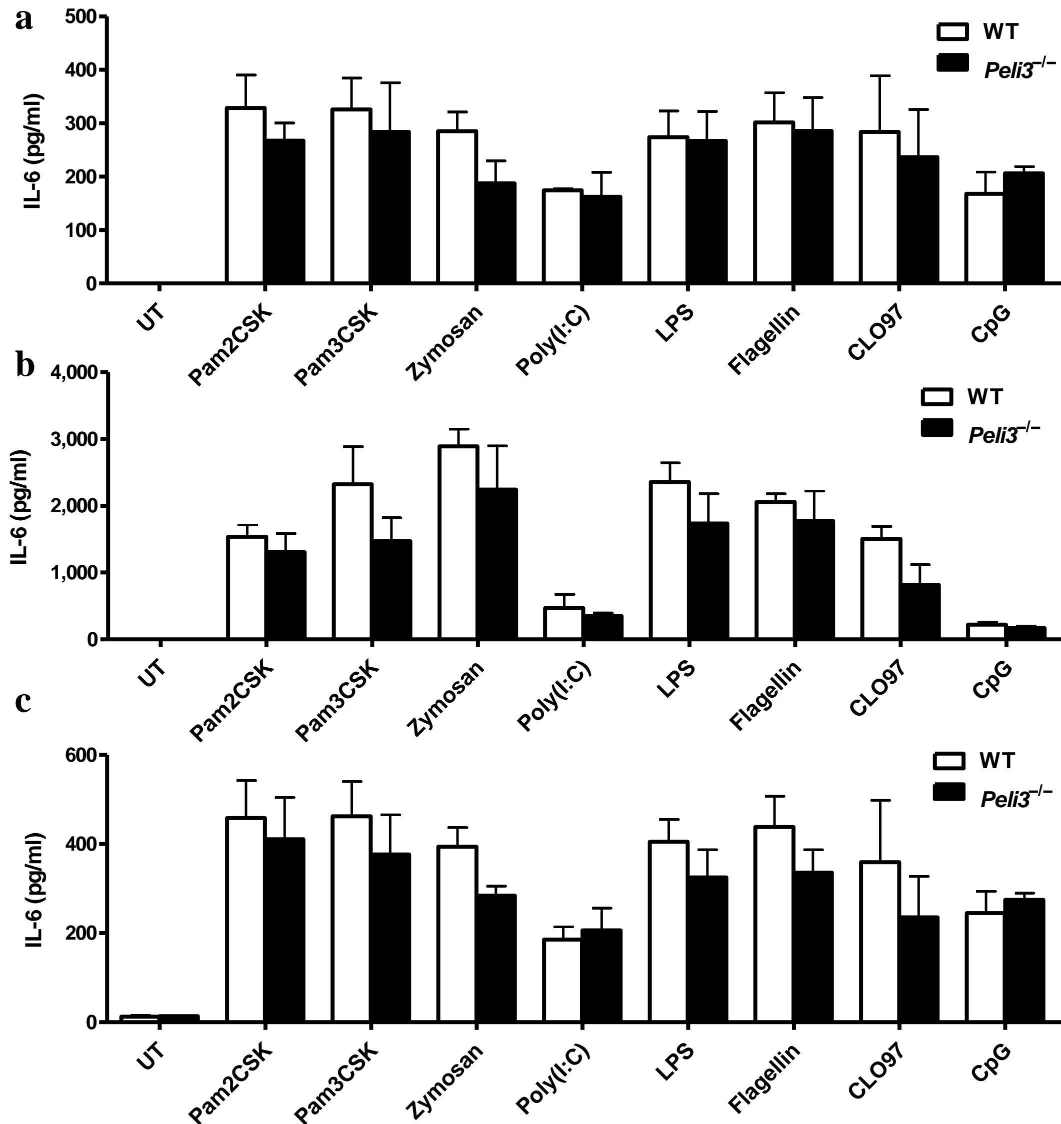


Figure 3.6 Effect of Pellino3-deficiency on TLR-induced production of IL-6.

ELISA of IL-6 production in media from **(a)** BMDMs or **(b)** BMDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Pam_2CSK (10 ng/ml), Pam_3CSK (10 ng/ml), Zymosan (5 μg/ml), Poly(I:C) (10 μg/ml), LPS (10 ng/ml), Flagellin (1 μg/ml), CLO97 (1 μg/ml) or CpG (3 μM) for 24 h. ELISA of TNF-α production in media from **(c)** MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Pam_2CSK (100 ng/ml), Pam_3CSK (100 ng/ml), Zymosan (5 μg/ml), Poly(I:C) (25 μg/ml), LPS (100 ng/ml), Flagellin (5 μg/ml), CLO97 (5 μg/ml) or CpG (5 μM) for 24 h. Higher concentrations of the each TLR ligand was used as MEFs are not as responsive to TLR activation as professional innate immune cells. Data are presented as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test.

3.2.5 Loss of Pellino3 Leads to Enhanced TLR3-induced Expression of IFN-β

Another critically important consequence for host immunity during TLR signalling is the activation of anti-viral type 1 IFNs. TLR3, TLR4, TLR7/8 and TLR9 are the predominant TLR family members that drive expression of type 1 IFNs while other TLRs have either limited or no ability to induce expression. Therefore, BMDMs (Fig. 3.7a) and BMDCs (Fig. 3.7b) from WT and *Peli3*$^{-/-}$ were stimulated for 24 h with ligands for TLR3, TLR4, TLR7/8, and TLR9, and their ability to induce IFN-β production was measured by ELISA. In addition, MEFs from WT and *Peli3*$^{-/-}$ mice were stimulated with Poly(I:C) for 24 h and IFN-β levels were also assessed by ELISA (Fig. 3.7c). Each of the ligands triggered IFN-β upregulation in the WT cells and in all cases, except the TLR3 ligand Poly(I:C), the levels of induction of IFN-β were comparable in the *Peli3*$^{-/-}$ cells. However, there was a significant augmentation of Poly(I:C)-induced expression of IFN-β induction in the *Peli3*$^{-/-}$ cells. This data indicates that Pellino3 is a specific regulator of TLR3-induced IFN-β in multiple cell types.

Studies were next performed to assess if the specific effects of Pellino3 on IFN-β expression manifested at the level of transcriptional regulation of the IFN-β expression. BMDMs (Fig. 3.8a) and BMDCs (Fig. 3.8b) from WT and *Peli3*$^{-/-}$ mice were thus stimulated with the ligands for TLR3, TLR4, TLR7/8 and TLR9 and measured for IFN-β mRNA expression by quantitative real-time PCR analysis. As was the case for protein levels of IFN-β, *Peli3*$^{-/-}$ cells showed greater induction of IFN-β mRNA in response to Poly(I:C), relative to WT cells suggesting that the ability of Pellino3 to modulate TLR3-induced expression of IFN-β is occurring at the transcriptional level.

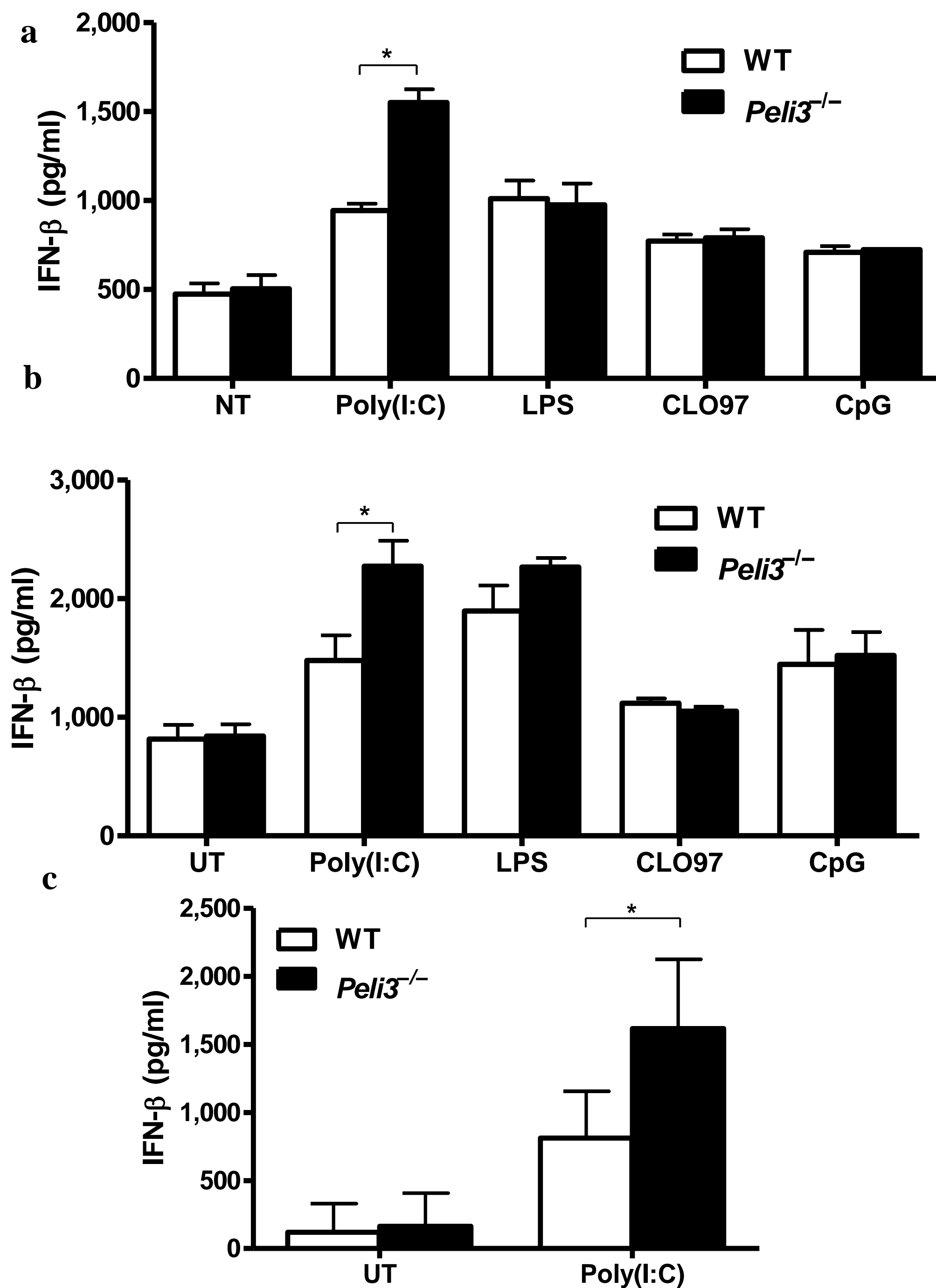


Figure 3.7 Effect of Pellino3-deficiency on TLR-induced production of IFN-β.

ELISA of IFN-β production in media from (**a**) BMDMs or (**b**) BMDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (10 μg/ml), LPS (10 ng/ml), CLO97 (1 μg/ml) or CpG (3 μM) for 24 h. ELISA of IFN-β production in media from MEFs (**c**) isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or Poly(I:C) (25 μg/ml) for 24 h. Data are presented as the mean +/- S.E.M. of at least three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$

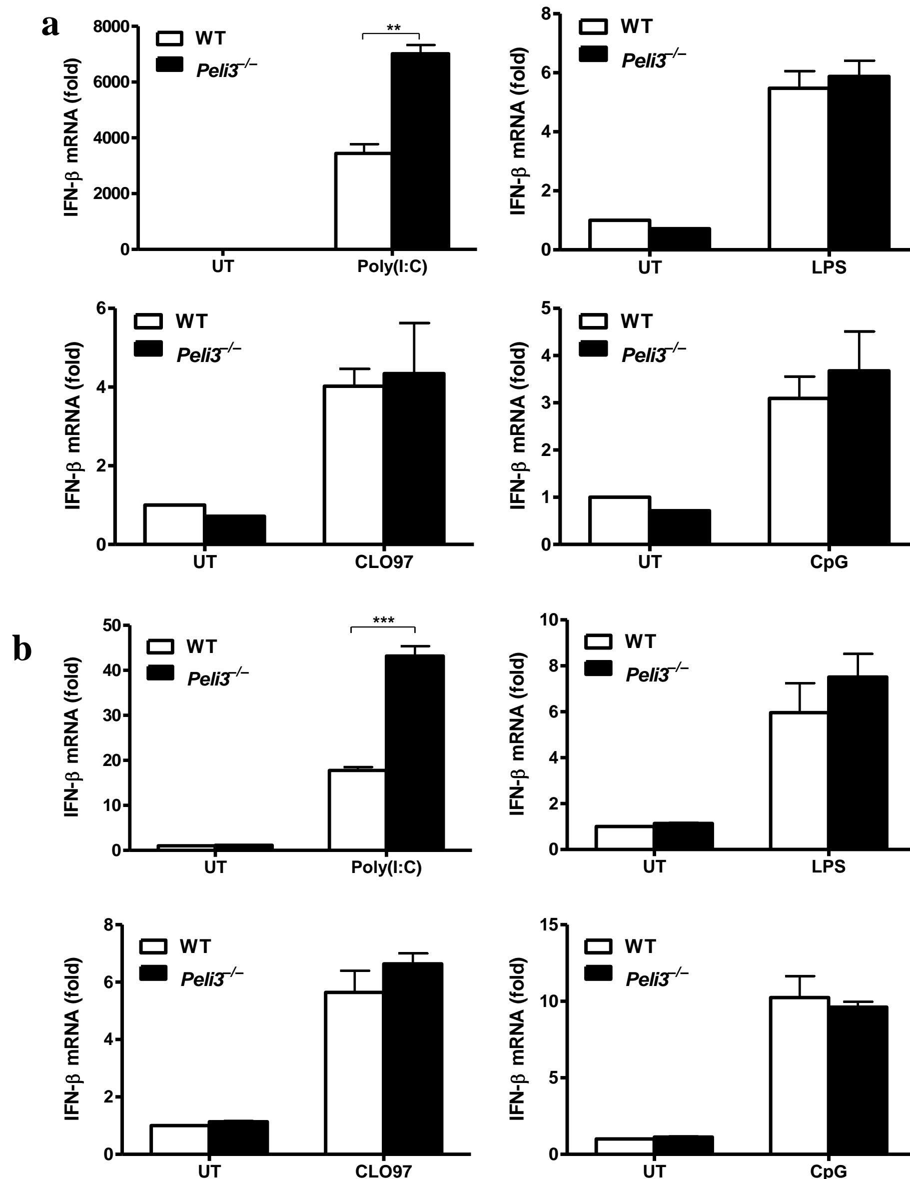


Figure 3.8 Effect of Pellino3-deficiency on TLR-induced expression of IFN-β mRNA.

Quantitative real-time PCR of IFN-β expression in cells from **(a)** BMDMs or **(b)** BMDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (10 μg/ml), LPS (10 ng/ml), CLO97 (1 μg/ml) or CpG (3 μM) for 6 h. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. **, $p < 0.01$; ***, $p < 0.001$.

3.2.6 Loss of Pellino3 Leads to Enhanced TLR3-induced Expression of IFN-α

Given that IFN-α is another type 1 IFN whose expression is transcriptionally regulated by a mechanism similar to IFN-β, the loss of Pellino3 affect on the expression of IFN-α was next examined. BMDMs (Fig. 3.9a), BMDCs (Fig. 3.9b) and MEFs (Fig. 3.9c) were isolated from WT and *Peli3*$^{-/-}$ mice stimulated for 24 h with Poly(I:C) and levels of IFN-α2/4 were measured by ELISA. Poly(I:C)-induced modest expression levels of IFN-α in all 3 cell types from WT mice but the corresponding cells from Pellino3-deficient mice showed much stronger induction of IFN-α protein production.

To confirm if Pellino3 was again targeting the transcriptional regulation of this type 1 IFN, and if it was again a TLR3 restricted phenomena, BMDMs (Fig. 3.10a) and BMDCs (Fig. 3.10b) from WT and *Peli3*$^{-/-}$ mice were stimulated with the ligands for TLR3, TLR4, TLR7/8, CLO97 and TLR9 for 6 h and mRNA measured by quantitative real-time PCR. In BMDMs from WT mice, all ligands induced IFN-α4 transcription with Poly (I:C) being the most efficacious. In addition, the Poly(I:C)-induced transcription of IFN-α4 was further enhanced in BMDMs from *Peli3*$^{-/-}$ mice. The ligands for TLR4, TLR7/8 and TLR9-induced low levels of IFN-α transcript in cells from WT mice but unlike Poly(I:C), the levels of IFN-α4-induced by these other ligands were the same in BMDMs from WT and Pellino3-deficent cells. The selective effects of Pellino3-deficiency on TLR3-induced IFN-α were also observed in BMDCs. In the latter cells, Poly(I:C) was most efficacious in inducing IFN-α4 transcription in cells from WT mice and again this was further increased in the Pellino3-deficient cells. As before, LPS and CLO97-induced IFN-α4 transcription in WT BMDCs and the levels of induced transcripts were similar in cells from *Peli3*$^{-/-}$ mice. Taken together, these findings highlight Pellino3 as specific negative regulator of type 1 IFNs during TLR3 signalling, likely mediating its inhibitory effects via regulation of gene transcription.

3.2.7 Pellino3 Limits Bioactive Type 1 IFN Secretion in Response to TLR3 Signalling

Given that loss of Pellino3 leads to enhanced expression of type 1 IFNs in response to TLR3 stimulation it was important to confirm that these IFNs were bioactive. To this end, BMDMs (Fig. 3.11a), BMDCs (Fig. 3.11b) and MEFs (Fig. 3.11c) from WT and *Peli3*$^{-/-}$ mice were stimulated with the TLR3 ligand Poly(I:C) for 24 h. Conditioned media from cells were incubated with a bioactive type 1 IFN blue sensor cell assay in which increased IFN signalling is measured as increased secretion of the reporter gene SEAP under the control of the ISG54 promoter. Secreted SEAP levels were measured quantitatively upon addition of Quanti-blue substrate and absorbance measured at 630 nm. In all 3 cell types, TLR3 activation resulted in an upregulation of bioactive type 1 IFNs and a further augmentation was detected in supernatants from Pellino3-deficent cells compared to their WT cell counterparts.

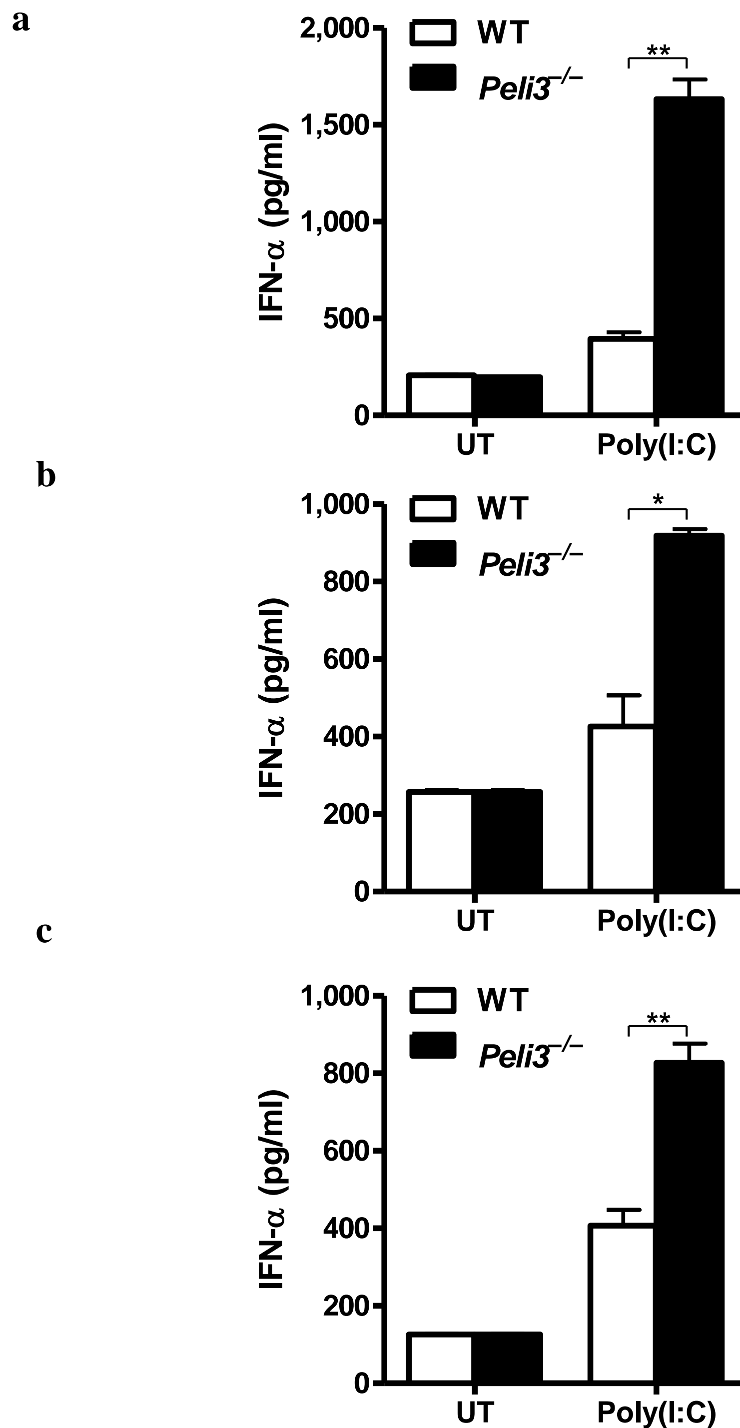


Figure 3.9 Effect of Pellino3-deficiency on TLR3-induced production of IFN-α.

ELISA of IFN-α production in media from (**a**) BMDMs or (**b**) BMDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (10 μg/ml). ELISA of IFN-α production in media from (**c**) MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or with Poly(I:C) (25 μg/ml) for 24 h. Data are presented as the mean +/- S.E.M. of at least three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

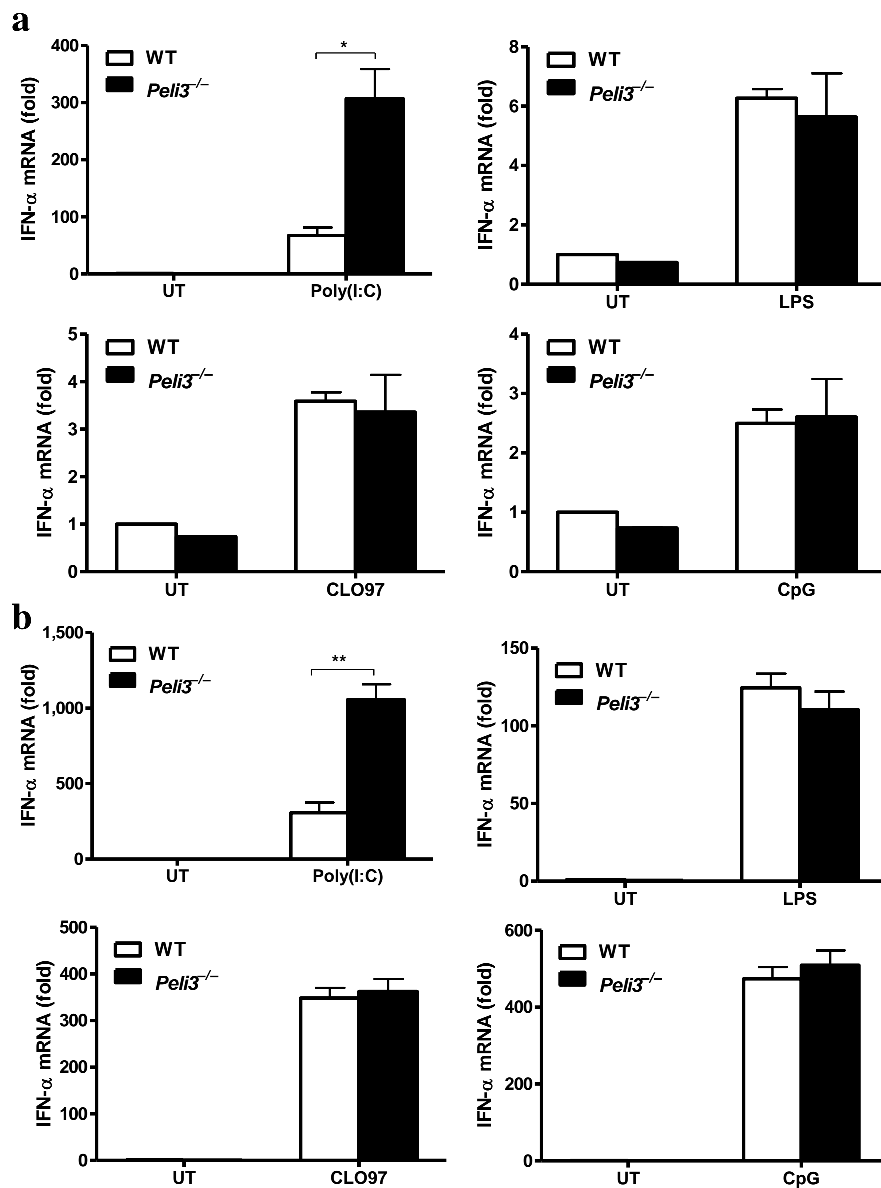


Figure 3.10 Effect of Pellino3-deficiency on TLR-induced expression of IFN-α mRNA.

Quantitative real-time PCR of IFN-α expression in cells from **(a)** BMDMs or **(b)** BMDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (10 μg/ml), LPS (10 ng/ml), CLO97 (1 μg/ml) or CpG (3 μM) for 6 h. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

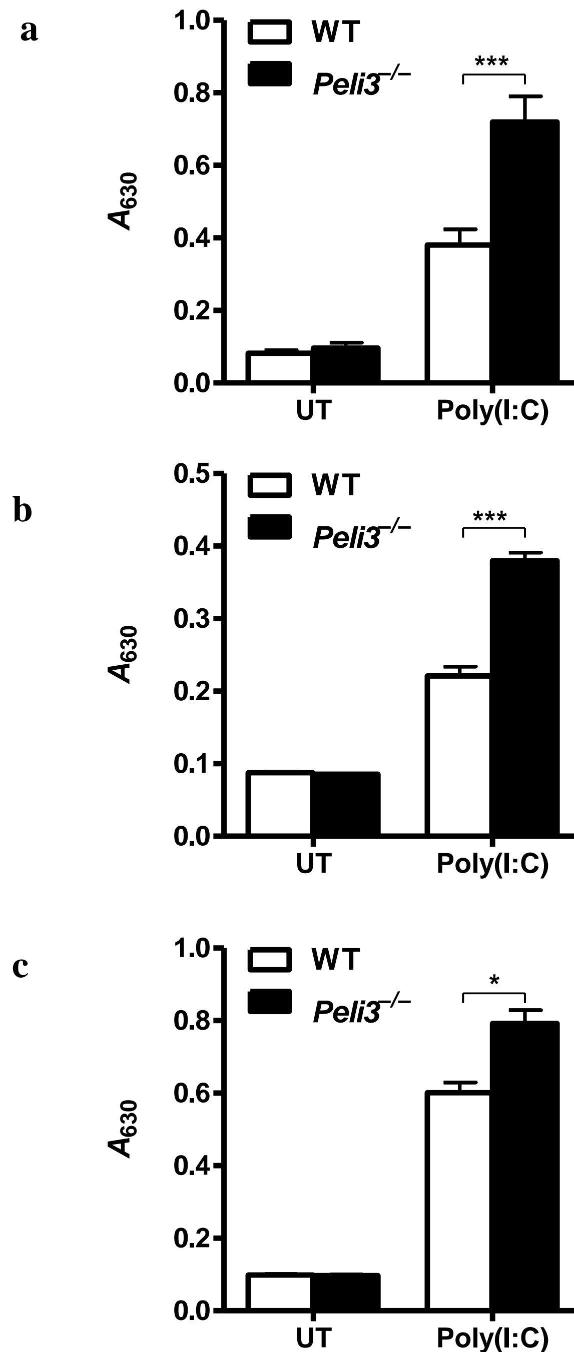


Figure 3.11 Effect of Pellino3-deficiency on TLR3-induced production of bioactive type 1 IFN.

Bioactive type 1 IFN blue sensor cell assay of media from **(a)** BMDMs or **(b)** BMDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (10 µg/ml). Bioactive type 1 IFN blue sensor cell assay in media from MEFs **(c)** isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (25 µg/ml) for 24 h. Data are presented as the absorbance at 630 nm and are the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$; ***, $p < 0.001$.

3.2.8 Pellino3 Regulates TLR3-induced Production of IP-10

In addition to type 1 IFNs, a number of other soluble mediators play fundamental roles in the clearance of viral pathogens. The chemokine IP-10 is thought to play an essential role in recruitment of various effector cells to limit viral replication during infection. The expression of IP-10 is also regulated by mechanisms similar to type 1 IFNs. BMDMs (Fig. 3.12a), BMDCs (Fig. 3.12b) and MEFs (Fig. 3.12c) were stimulated with TLR3, TLR4, TLR7/8 and TLR9 ligands for 24 h and IP-10 levels assessed by ELISA. Each stimulation resulted in an increase in IP-10 production in the different cells types. Similar levels of the chemokine were observed in response to TLR4, TLR7/8 and TLR9 stimulations between WT and Pellino3-deificent cells, however, a specific augmentation in IP-10 production was detected in Pellino3-defienct cells upon TLR3 activation compared to WT cells.

To ensure Pellino3 was targeting IP-10 production in a similar fashion to that of the type 1 IFNs, the effect of Pellino3 deficiency on IP-10 transcription was next investigated. BMDMs (Fig. 3.13a) and BMDCs (Fig. 3.13b) from WT and *Peli3*$^{-/-}$ were stimulated with the ligands for TLR3, TLR4, TLR7/8 and TLR9 for 6 h and mRNA was extracted. Real-time PCR analysis for IP-10 indicated that Pellino3 was once again selectively targeting the transcription of the anti-viral gene during TLR3 signalling as the Poly(I:C) stimulated cells deficient in Pellino3 showed significant augmentation in IP-10 expression relative to WT cells. This data taken together strongly indicate that Pellino3 is specifically targeting the TLR3 antiviral signalling pathway.

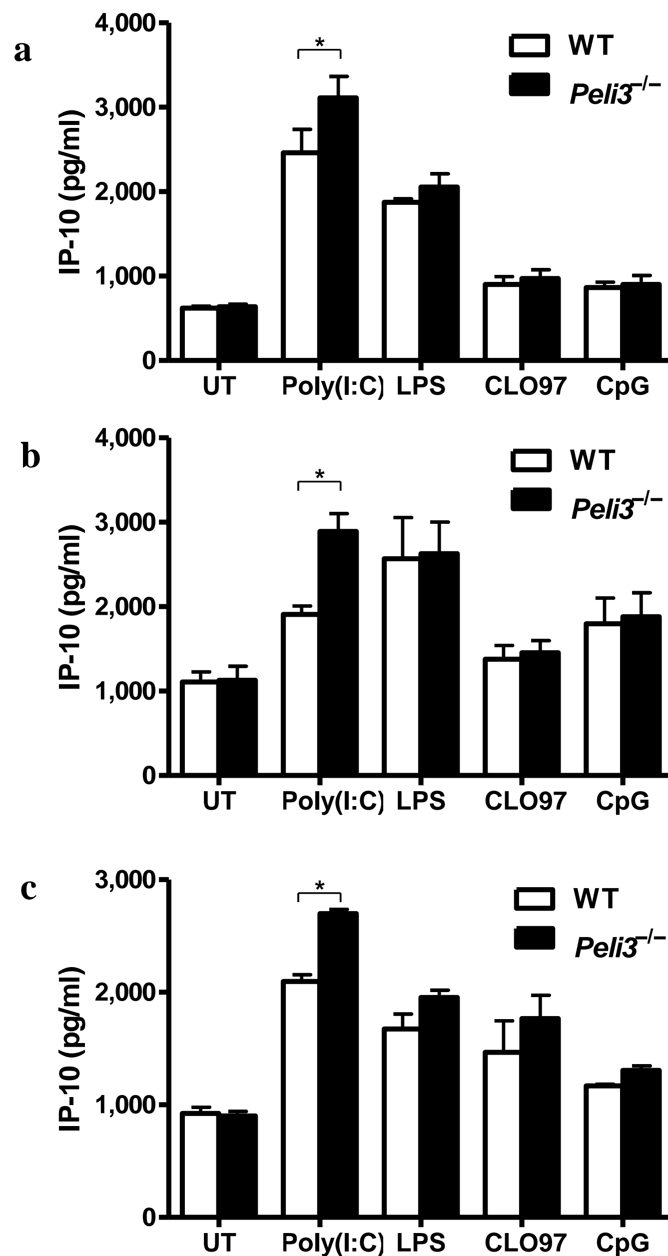


Figure 3.12 Effect of Pellino3-deficiency on TLR-induced production of IP-10.

ELISA of IP-10 production in media from **(a)** BMDMs or **(b)** BMDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (10 μg/ml), LPS (10 ng/ml), CLO97 (1 μg/ml) or CpG (3 μM) for 24 h. ELISA of IP-10 production in media from **(c)** MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (25 μg/ml), LPS (100 ng/ml), CLO97 (5 μg) or CpG (5 μM) for 24 h. Data are presented as the mean +/- S.E.M. of at least three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$.

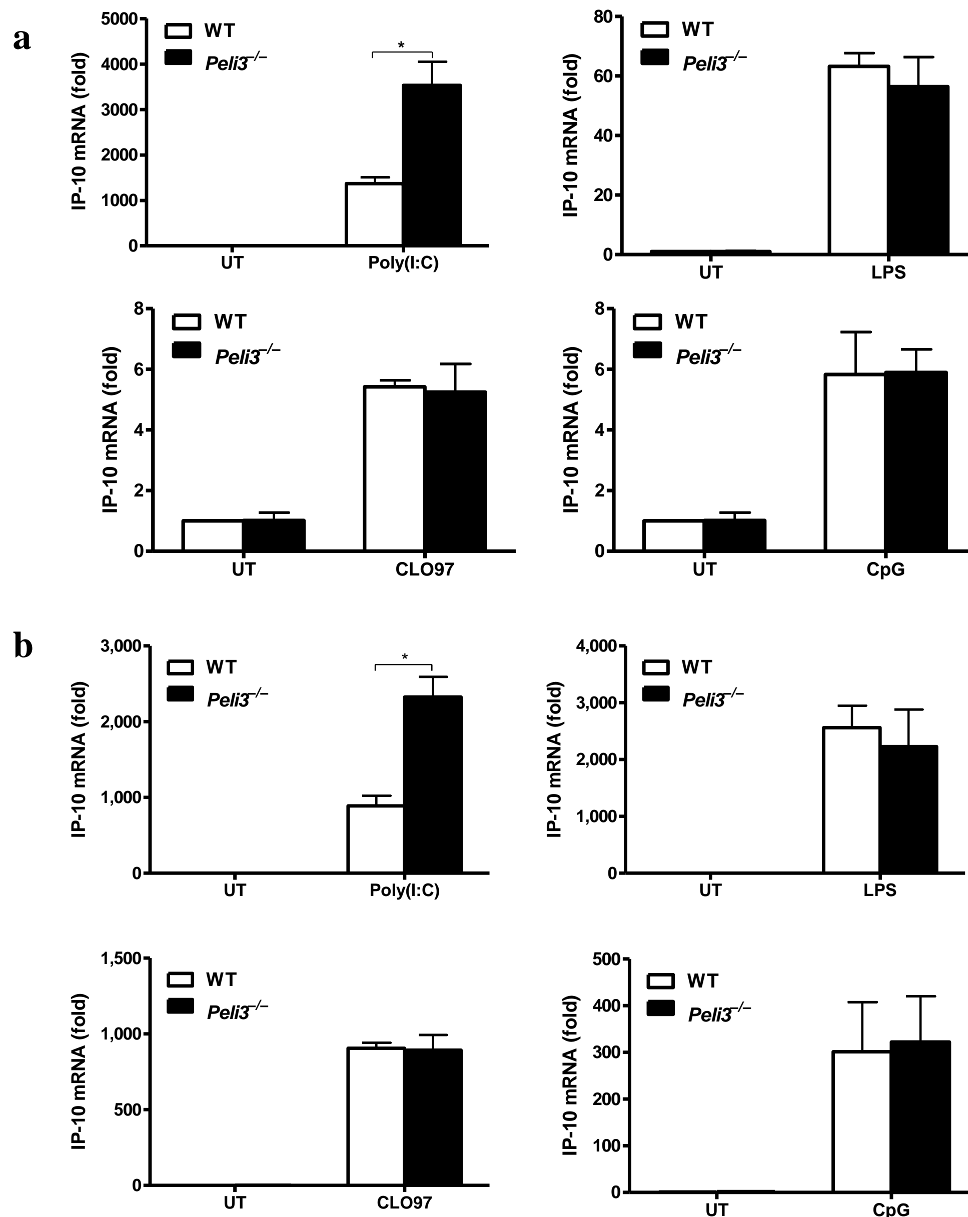


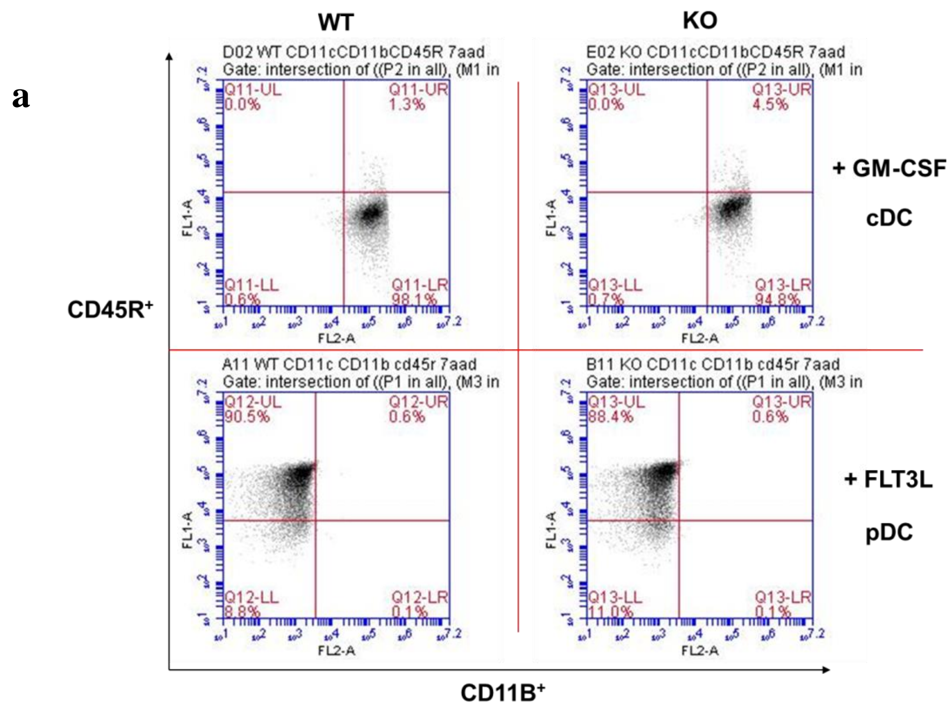
Figure 3.13 Effect of Pellino3-deficiency on TLR-induced expression of IP-10.

Quantitative real-time PCR of IP-10 expression in cells from **(a)** BMDMs or BMDCs **(b)** isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (10 µg/ml), LPS (10 ng/ml), CLO97 (1 µg) or CpG (3 µM) for 6 h. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$.

3.2.9 Pellino3 does not Regulate the Expression of Type 1 IFNs in Response to TLR Stimulation in pDCs.

A subset of dendritic cells, the pDCs are known to be specialised and rapid producers of type 1 IFNs in response to viral infections. Interestingly, the pDCs detect viral RNA and DNA using only endosomal TLR7/8 and TLR9 and have no expression of TLR3. As Pellino3 is a specific regulator of TLR3-induced anti-viral signalling, it was of importance to investigate if Pellino3 could mediate its regulatory effects in cells that respond to viral PAMPs by producing type 1 IFNs in a TLR3-independent manner. To this end, cells from WT and *Peli3*$^{-/-}$ mice bone marrow was driven to both a BMDC (with GM-CSF) and pDC (with FLT3L) cell lineage for 9 days. After polarisation, the pDC cells were subject to a negative selection enrichment process using antibodies conjugated to magnetic beads that label non pDC cells for retention in a magnetic column allowing a purified population of pDC cells to be obtained. Flow cytometry analysis (Fig. 3.14a) was used to assess the purity of cell populations. Live viable cells which stained negative for 7aad and positive for the pan DC marker CD11c were gated upon and used for dot plots provided. Cells that stained positive for CD11b and negative for CD45R (lower right quadrant) were considered BMDCs. With purity of 98.1% and 94.8% in the WT and *Peli3*$^{-/-}$ cells respectively, after GM-CSF-induced polarisation, it indicates that the absence of Pellino3 has no effect on the percentage of BMDCs generated by GM-CSF. Cells that stained CD11b negative and CD45R positive (upper left quadrant) were considered pDCs. The enriched cell population from WT and *Peli3*$^{-/-}$ pDCs were also comparable with purities of 90.5% and 88.4% respectively. Using these enriched pDC populations, WT and *Peli3*$^{-/-}$ (Fig. 3.14b) pDCs were stimulated with the ligands for TLR3, TLR4, and TLR9 and their ability to produce IL-6, IP-10, IFN-β and IFN-α was measured by ELISA. In line with other published reports, TLR3 and TLR4 stimulation failed to induce cytokines in the pDCs. Stimulation of TLR9 in WT pDCs resulted in robust induction of IL-6, IP-10, IFN-β and IFN-α with WT and Pellino3-deficent cells displaying comparable levels of cytokine production. This data indicates Pellino3 regulation of type 1 IFNs is specific to TLR3 signalling.

a



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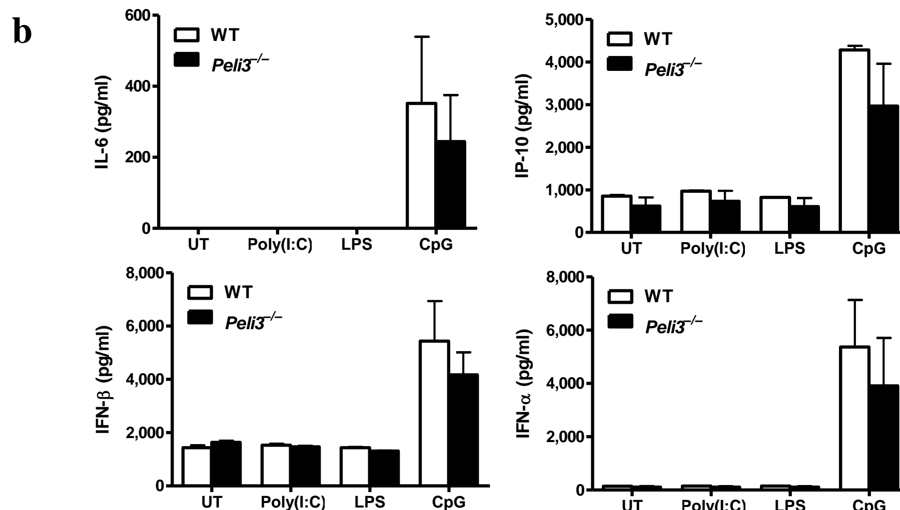

Figure 3.14 Effect of Pellino3-deficiency on expression of TLR-responsive induced cytokines in pDCs.

(a) Generation of BMDCs and pDCs. BMDCs and pDCs were generated from bone marrow cells in the presence of GM-CSF (10 ng/ml) or FLT3L (10 ng/ml) respectively for 9 days with media changed at 3 day intervals. The pDCs were subject to magnetic bead enrichment prior to flow cytometry analysis. Dot plots shown are gated on CD11c positive cells that are viable and stained 7aad negative. CD11b positive and CD45R negative cells were considered BMDCs while CD11b negative and CD45R positive cells were considered pDCs. (**b**) ELISA of IL-6, IP-10, IFN-β and IFN-α production in media from pDCs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (10 µg/ml), LPS (10 ng/ml) or CpG (3 µM) for 24 h. Data are presented as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test.

3.2.10 Pellino3 does not Target the MDA5 Pathway

Given that Poly(I:C) can potentially stimulate both TLR3 and the cytosolic receptor MDA-5, studies next probed which receptor pathway was being targeted by Pellino3 in its modulation of cell responsiveness to Poly(I:C). BMDMs from WT and *Peli3*$^{-/-}$ mice were stimulated directly with Poly(I:C) to favour TLR3 activation or subjected to liposome-mediated transfection with Poly(I:C), to facilitate its delivery to the cytosolic MDA5 receptors (Fig. 3.15a). Both delivery methods induced IFN-β in the WT BMDMs, and this was further enhanced in Pellino3-deficient cells when the Poly(I:C) was directly applied to the cells but not when transiently transfected. This further supported the earlier proposal that Pellino3 is targeting the TLR3 pathway. This was further validated by showing that responses to Poly(I:C) in the present models were mediated by TLR3 and not Mda-5 (Fig. 3.15b). Thus immortalised BMDMs derived from WT, TRIF- and MAVS-deficient mice were treated with Poly(I:C). Quantitative real-time PCR for IFN-β revealed a loss of induction only in the TRIF-deficient cells with strong activation in WT and M*avs*$^{-/-}$ macrophages. Given that TRIF is a critical adaptor for TLR3 and MAVS critical for MDA5 signal propagation, these data confirm that the presently used direct application of Poly(I:C) to cells is activating the TLR3 pathway and not Mda-5 and by extension the regulatory effects of Pellino3 on Poly(I:C) signalling are due to targeting of the TLR3 pathway.

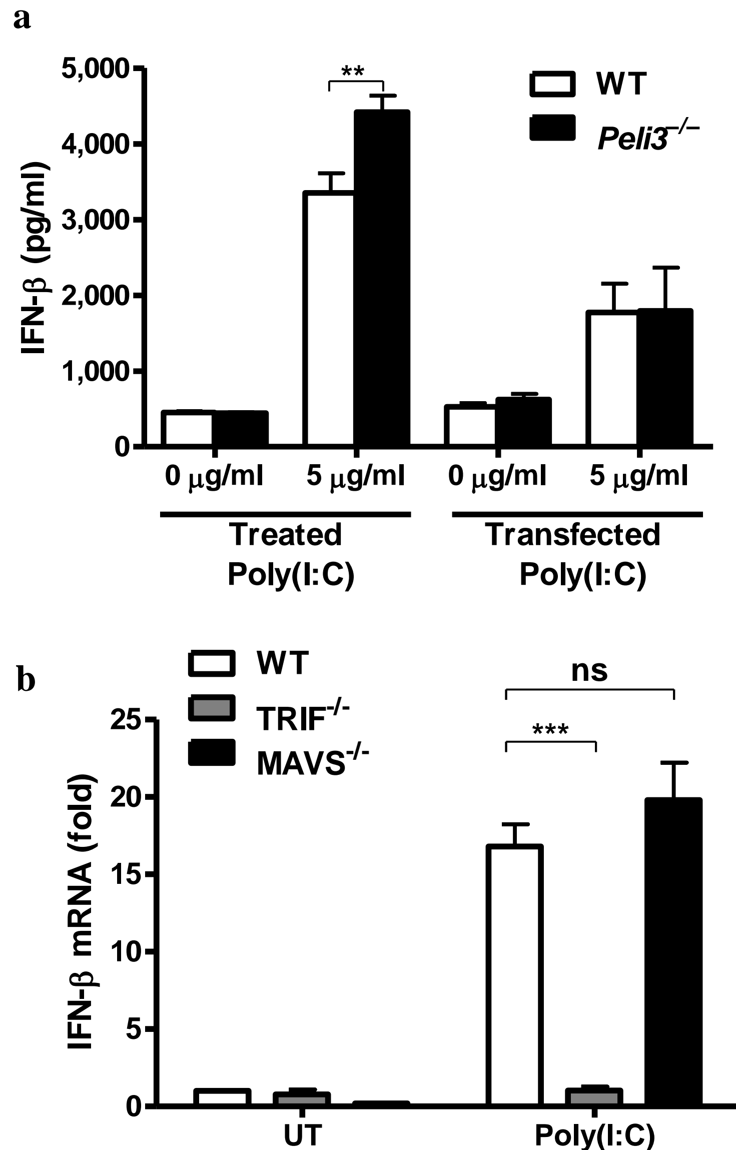

Figure 3.15 Differential effects of Pellino3-deficiency on TLR3 and MDA5 signalling.

(a) ELISA of IFN-β protein in media from BMDMs from WT and *Peli3*$^{-/-}$ mice and left untreated (UT), stimulated with Poly(I:C) (5 μg/ml) or transfected with Poly(I:C) (5 μg/ml) for 24 h. **(b)** Quantitative real-time PCR of IFN-β mRNA expression in immortalised WT, *Trif*$^{-/-}$ and *Mavs*$^{-/-}$ BMDMs untreated (UT) or stimulated with Poly(I:C) (25 μg/ml) for 6 h. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. **, $p < 0.01$; ***, $p < 0.001$; ns, not significant.

3.2.11 Pellino3 Expression is TLR3 Responsive

As a number of negative regulators of innate immune signalling are functionally upregulated by the signals they inhibit, it was important to investigate if Pellino3 expression was TLR3 responsive. Immortalised BMDMs derived from WT, *TRIF*$^{-/-}$, *TBK1*$^{-/-}$ and *MAVS*$^{-/-}$ were stimulated with Poly(I:C) for 6 h and mRNA extracted (Fig. 3.16a). Poly(I:C)-induced the expression of Pellino3 mRNA in WT BMDMs however, its upregulation was significantly reduced in the *TRIF*$^{-/-}$ and *TBK1*$^{-/-}$ BMDMs. Interestingly, cells deficient in MAVS, the critical adaptor for MDA5 signalling, had no significant impact on Poly(I:C)-induced levels of Pellino3 mRNA suggesting that Pellino3 mRNA expression is specifically regulated by TLR3 signalling. Given that Poly(I:C) is a synthetic analogue of viral dsRNA, the role of a live virus in upregulating Pellino3 expression was next explored. EMCV was chosen as during its infection cycle it generates dsRNA and TLR3 signalling has been shown to be important for host protection (Hardarson *et al.*, 2007b). Immortalised BMDMs derived from WT, TRIF$^{-/-}$, TBK1$^{-/-}$ and MAVS$^{-/-}$ were infected with live EMCV for 25 h and mRNA extracted (Fig. 3.16b). EMCV infection caused potent upregulation of Pellino3 mRNA in WT iBMDMs. Again, the absence of TRIF or TBK1 leads to significant abrogation of viral-induced Pellino3 expression. Importantly, MAVS deficiency had no significant effect on Pellino3 upregulation. As EMCV infection activates TLR3 and MDA5, this data strongly indicates that Pellino3 is targeting the TLR3 pathway during EMCV infection.

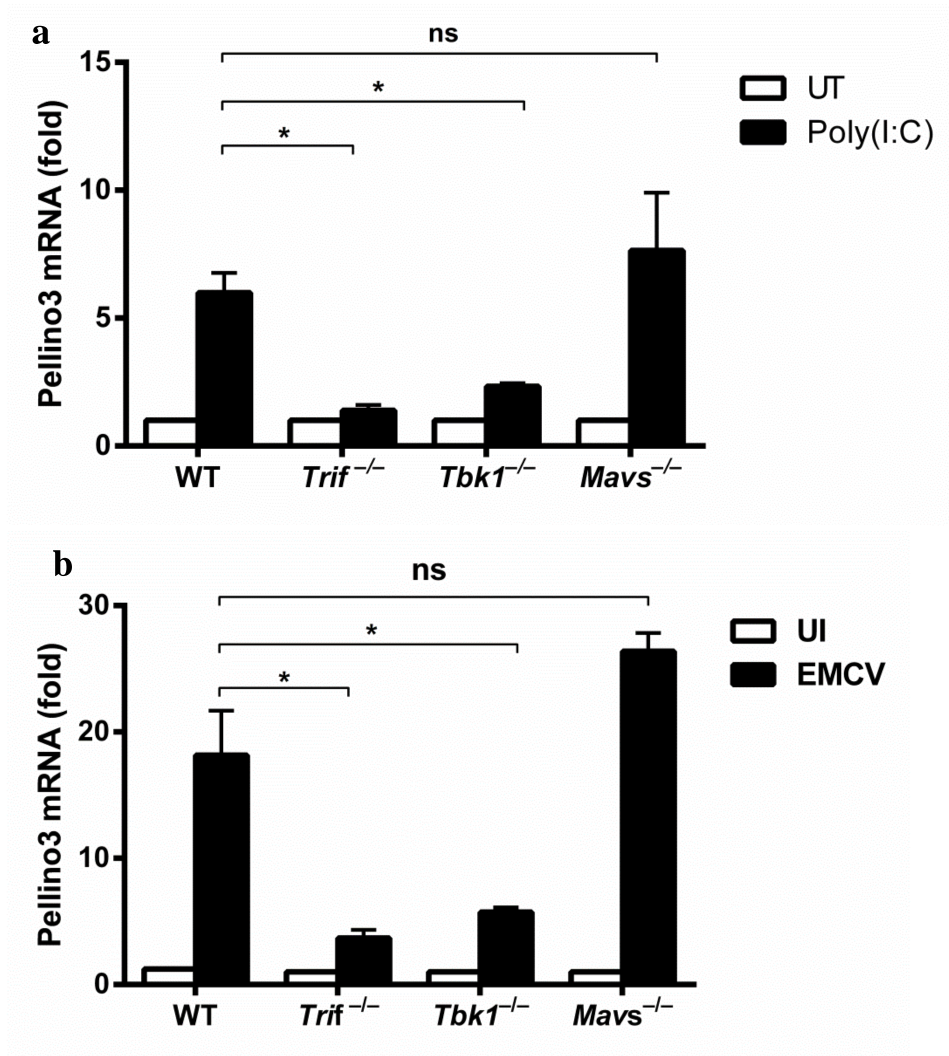

Figure 3.16 TLR3 induces Pellino3 expression in a TRIF pathway-dependent manner

Quantitative real-time PCR of Pellino3 expression in immortalised BMDMs and left **(a)** untreated (UT) or stimulated with Poly(I:C) (25 μg/ml) for 6 h or **(b)** left uninfected (UI) or infected with live EMCV (250 PFU) for 25 h. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$; ns, not significant.

3.2.12 Pellino3 Negatively Regulates EMCV-induced Expression of IFN-β

Although EMCV is capable of driving Pellino3 expression, it was important to demonstrate that Pellino3 could also negative regulate the anti-viral response during a live EMCV infection. BMDMs (Fig. 3.17a) and MEFs (Fig. 3.17b) were isolated from WT and *Peli3*$^{-/-}$ mice and were infected with live EMCV for 25 h and IFN-β production measured by ELISA. EMCV infection resulted in a potent upregulation of IFN-β in WT and Pellino3-deficent cells. EMCV induction of IFN-β was significantly enhanced in the Pellino3-deficient cells compared to that of WT cells. The transcription of IFN-β mRNA in BMDMs (Fig. 3.17c) and MEFs (Fig. 3.17d) from WT and *Peli3*$^{-/-}$ mice was also upregulated by EMCV infection. Again a further enhancement of the cytokine was evident in cells from Pellino3-deficent mice compared to WT cells. Together, this data indicates that Pellino3 can not only control Poly(I:C)-induced IFN-β but also plays a physiological role in regulating the expression of IFN-β in response to live viral challenge.

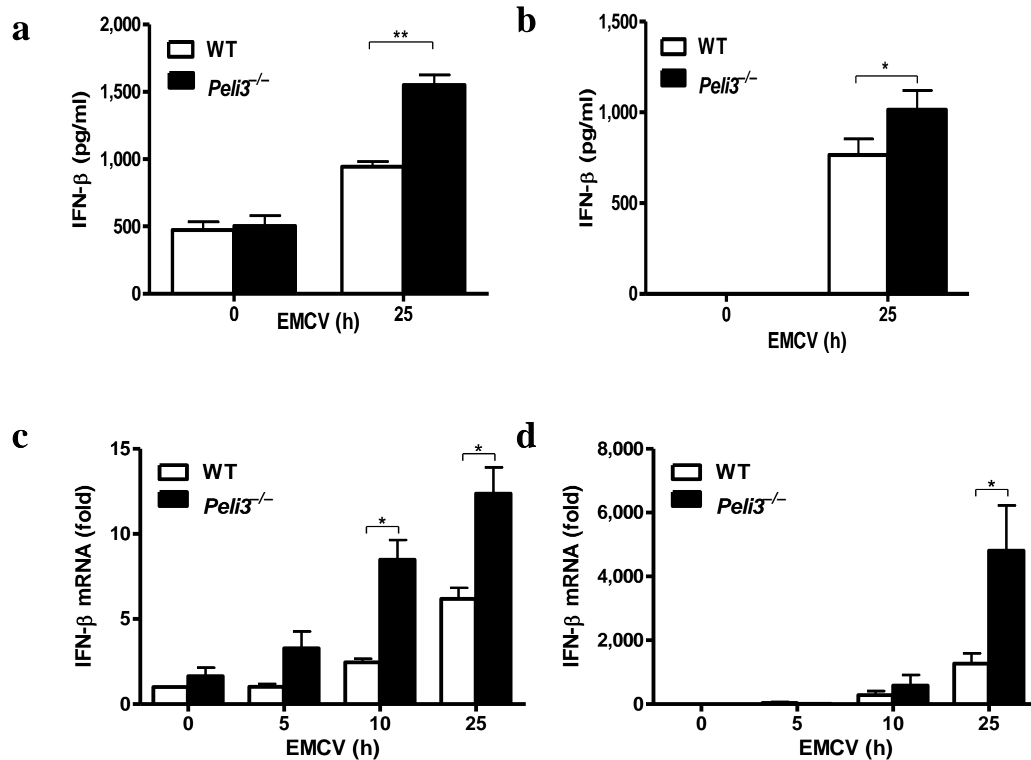

Figure 3.17 Effect of Pellino3-deficiency on EMCV-induced expression of IFN-β.

ELISA of IFN-β protein in media from **(a)** BMDMs or **(b)** MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left uninfected (UI) or infected with live EMCV (250 PFU). Quantitative real-time PCR of IFN-β expression in cells from **(c)** BMDMs or **(d)** MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left UI or infected with EMCV (250 PFU) for the indicated times. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test . *, $p < 0.05$; **, $p < 0.01$.

3.2.13 Pellino3 is a Negative Regulator of EMCV-induced IFN-α

To investigate if EMCV-induced other type 1 IFNs and if they were similarly regulated by Pellino3, BMDMs (Fig. 3.18a) and MEFs (Fig. 3.18b) were isolated from WT and *Peli3*$^{-/-}$ mice and were infected with live EMCV (250 PFU) for 25 h and IFN-α2/4 production measured by ELISA. Infected cells displayed a robust upregulation of IFN-α production in both WT and Pellino3-deficent cells. EMCV induction of IFN-α was significantly enhanced in the Pellino3-deficient cells compared to WT cells. Real-time PCR analysis investigating the transcription of IFN-α4 in WT and *Peli3*$^{-/-}$ mice BMDMs (Fig. 3.18c) and MEFs (Fig. 3.18d) revealed a similar phenotype in response to EMCV infection In BMDMs and MEFs significant augmentation of IFN-α4 mRNA levels was evident in Pellino3-deficent cells after 25 h infection compared to their WT counterparts. Therefore, Pellino3 has the ability to regulate other type 1 IFNs in response to viral infection.

3.2.14 Pellino3 Negatively Regulates EMCV-induced Bioactive type 1 IFN Secretion

In order for type 1 IFNs to mediate protection from EMCV virus, they must be able to functionally upregulate ISGs to help control viral replication. To this end, BMDMs (Fig. 3.19a) and MEFs (Fig. 3.19b) from WT and *Peli3*$^{-/-}$ mice were infected with live EMCV for 25 h and supernatants measured for expression of bioactive type 1 IFNs. Conditioned media from cells were incubated with the bioactive type 1 IFN blue sensor cell assay. EMCV infected cells displayed an increase in secreted bioactive type 1 IFNs. However, the levels of bioactive type 1 IFNs was further augmented in cells deficient in Pellino3 compared to WT cells highlighting Pellino3 as a functional regulator of type 1 IFN signalling in response to EMCV infection.

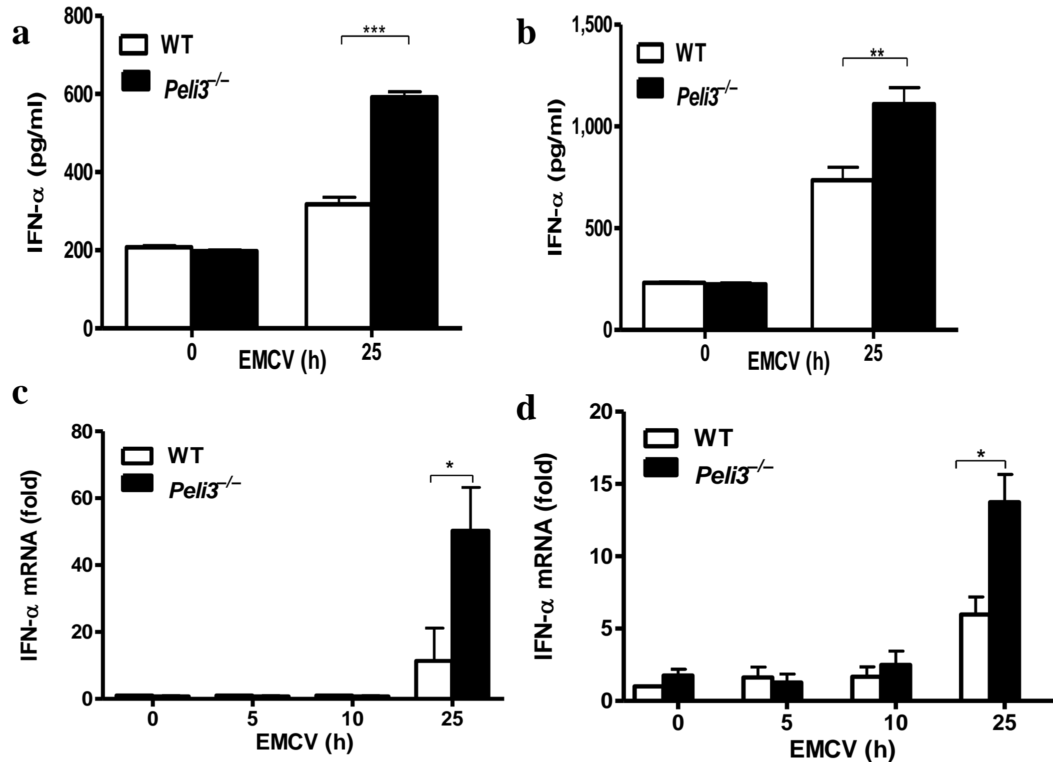

Figure 3.18 Effect of Pellino3-deficiency on EMCV-induced expression of IFN-α.

ELISA of IFN-α protein in media from **(a)** BMDMs or **(b)** MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left uninfected (UI) or infected with live EMCV (250 PFU). Quantitative real-time PCR of IFN-α expression in cells from **(c)** BMDMs or **(d)** MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left UI or infected with EMCV (250 PFU) for the indicated times. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test . *, $p < 0.05$; **, $p < 0.01$.

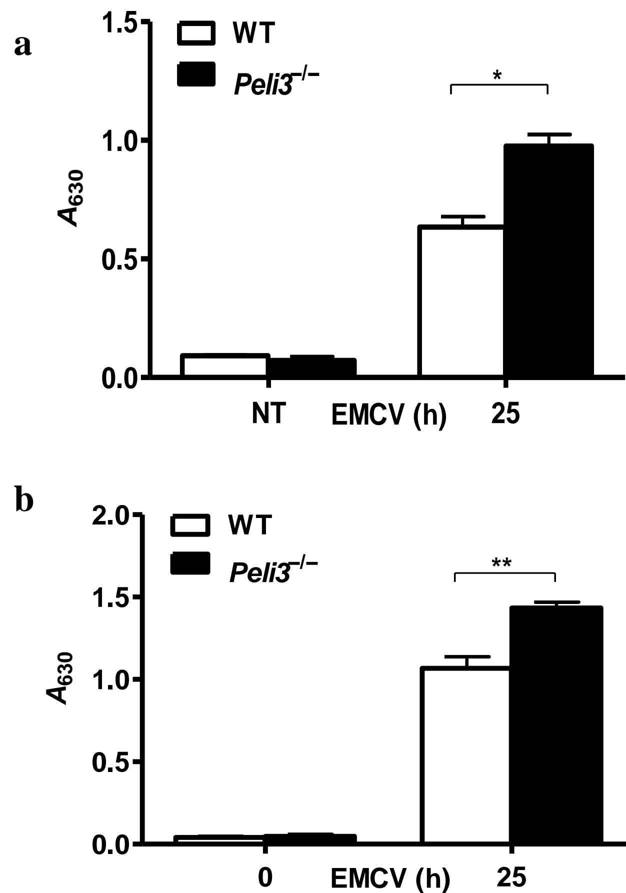

Figure 3.19 Effect of Pellino3-deficiency on EMCV-induced production of bioactive type 1 IFN.

Bioactive type 1 IFN blue sensor cell assay of media from **(a)** BMDMs or **(b)** MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (UT) or infected with EMCV (250 PFU) for 25 h. Data are presented as the absorbance at 630 nm and are the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

3.2.15 Pellino3 is a Negative Regulator of EMCV-induced IP-10 Expression

Investigation into IP-10 was again employed to investigate if Pellino3 affected other EMCV-responsive genes that are regulated in a manner similar to type 1 IFNs. BMDMs (Fig. 3.20a) and MEFs (Fig. 3.20b) were isolated from WT and *Peli3*$^{-/-}$ mice and infected with live EMCV for 25 h and IP-10 production measured by ELISA. EMCV-induced production of IP-10 in both WT and Pellino3-deficent cells however, EMCV induction of IP-10 was significantly enhanced in the Pellino3-deficient cells compared to WT cells. Real-time PCR analysis investigating the transcription of IP-10 in WT and *Peli3*$^{-/-}$ mice BMDMs (Fig. 3.20c) and MEFs (Fig. 3.20d) revealed a similar phenotype. In BMDMs and MEFs significant augmentation of IP-10 mRNA levels were evident in cells lacking Pellino3 expression compared to WT cells after 25 h infection. Therefore, Pellino3 has the ability to regulate other cytokines that share similar transcription factor regulation profiles to the type 1 IFNs.

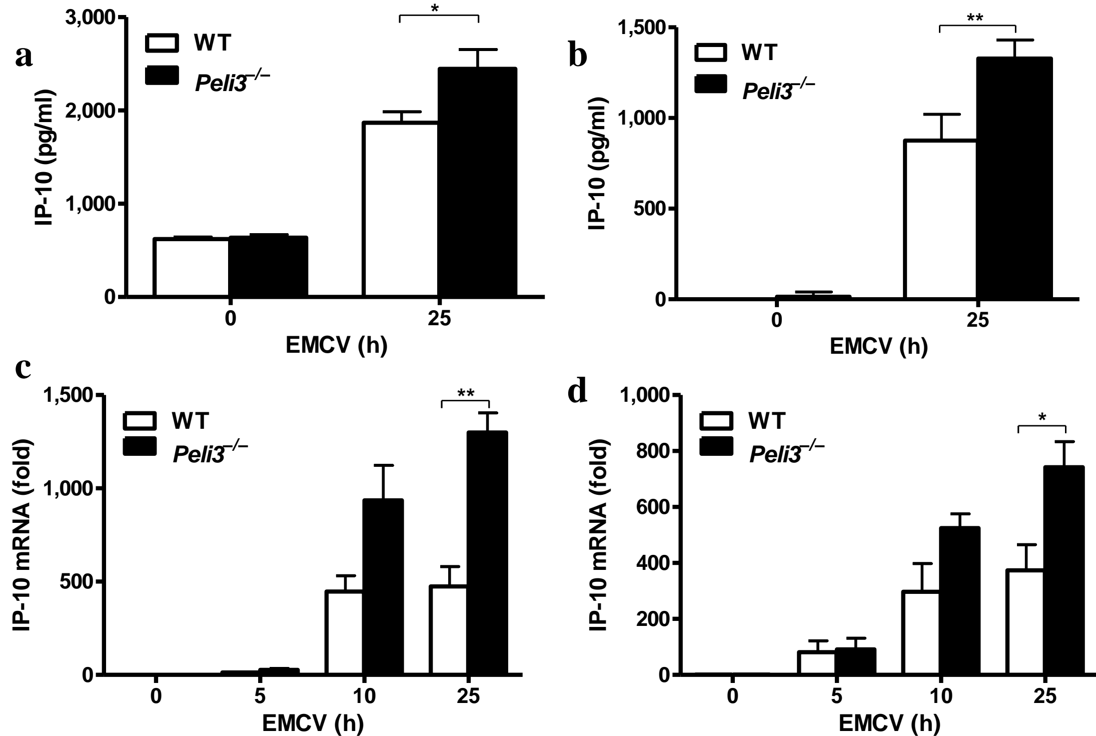

Figure 3.20 Effect of Pellino3-deficiency on EMCV-induced expression of IP-10.

ELISA of IP-10 protein in media from (**a**) BMDMs or (**b**) MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left uninfected (UI) or infected with live EMCV (250 PFU). Quantitative real-time PCR of IP-10 expression in cells from (**c**) BMDMs or (**d**) MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left UI or infected with EMCV (250 PFU) for the indicated times. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test . *, $p < 0.05$; **, $p < 0.01$.

3.2.16 Loss of Pellino3 Fails to Affect EMCV-induced TNF-α Production

To investigate whether Pellino3 was a specific regulator of viral-induced anti-viral cytokines or if it could also regulate the pro-inflammatory cytokine TNF-α, BMDMs (Fig. 3.21a) and MEFs (Fig. 3.21b) were isolated from WT and *Peli3*$^{-/-}$ mice and were infected with live EMCV for 25 h and TNF-α production measured by ELISA. EMCV-induced a modest activation of TNF-α in WT cells and this was also observed in cells from the *Peli3*$^{-/-}$ animals. EMCV infection induced the transcription of TNF-α in BMDMs (Fig. 3.21c) and MEFs (Fig. 3.21d) from WT and *Peli3*$^{-/-}$ mice, however no significant differences in the cytokine production were observed between WT and Pellino3-deficent cells. In BMDMs, EMCV-induced a time dependent increase in TNF-α mRNA expression until 10 h infection which began to be downregulated by 25 h. In MEFs, a time dependent increase in transcript was measured up until 25 h. This data indicates that EMCV regulation may undergo differential regulation in a cell type and temporal manner; however Pellino3 plays no role in these processes. The data to this point has provided definitive evidence that Pellino3 is a key and specific mediator of TLR3- and viral-induced type 1 IFNs in cells isolated ex-vivo from Pellino3 knockout mice.

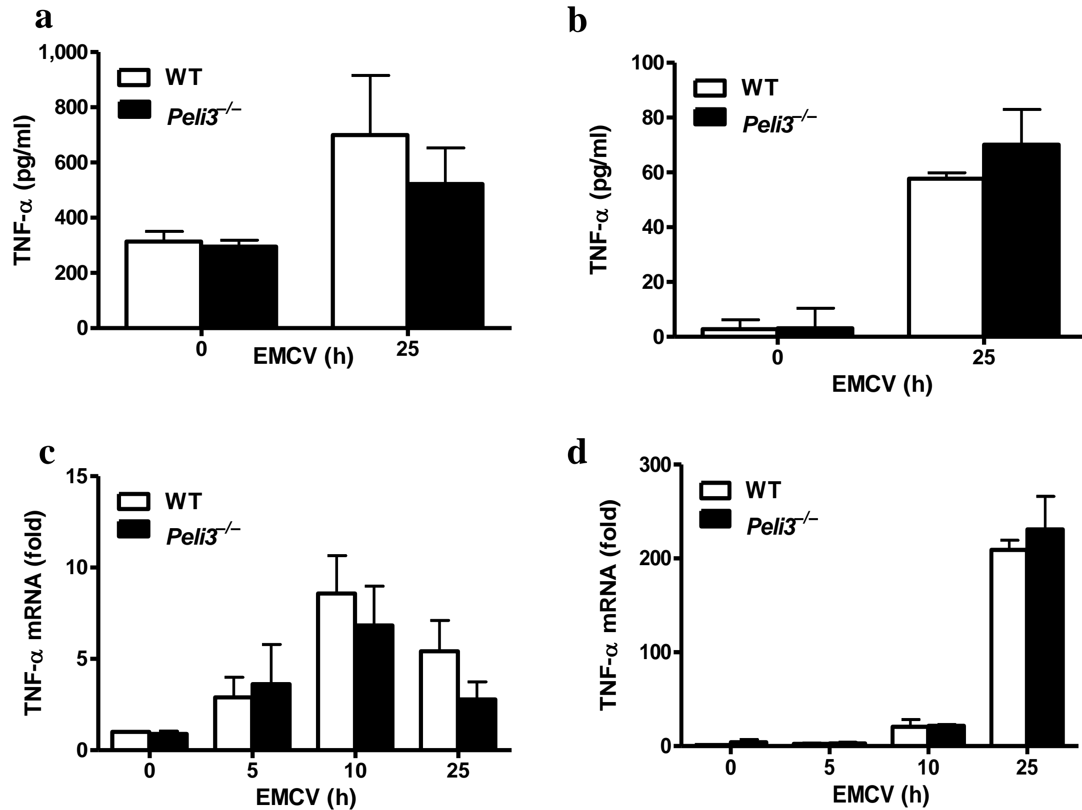

Figure 3.21 Effect of Pellino3-deficiency on EMCV-induced TNF-α.

ELISA of TNF-α production in media from (**a**) BMDMs or (**b**) MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left uninfected (UI) or infected with live EMCV (250 PFU). Quantitative real-time PCR of TNF-α expression in cells from (**c**) BMDMs or (**d**) MEFs isolated from WT and *Peli3*$^{-/-}$ mice and left UI or infected with EMCV (250 PFU) for the indicated times. Data are presented as the mean +/- S.E.M. of at least three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.2.17 Absence of Pellino3 Enhances Host Protection against EMCV Infection

The physiological and pathological relevance of these regulatory effects of Pellino3 in the context of an *in vivo* EMCV infection model was then investigated. Two lethal doses, 250 PFU (Fig. 3.22a left panel) and 50 PFU (Fig.3.22a right panel) of EMCV were injected intra-peritoneally into WT and *Peli3*$^{-/-}$ mice. The higher infection challenge had a lethal effect on all WT mice by 7 d while 60% of the *Peli3*$^{-/-}$ mice survived up until the end point of the experiment, 12 d. The lower dose of the virus triggered lethality in 50% of the WT mice with all the *Peli3*$^{-/-}$ mice surviving till the experimental end point. In order to assess what was happening physiologically during the infection, another cohort of animals were infected with 50 PFU for 4 days and sacrificed. An increased heart to body weight ratio, a hallmark of EMCV infection and associated with lethality was evident in the WT mice but not in the *Peli3*$^{-/-}$ mice (Fig. 3.22b). A blinded pathological examination of heart tissue from EMCV-infected animals by microscopy revealed histopathological changes with random multifocal mononuclear interstitial infiltrates and myofiber nuclear karyolysis and myofiber cell loss accompanied by mild edema (Fig. 3.22c). Such pathological alterations from control mice were not detected in infected *Peli3*$^{-/-}$ mice. The enhanced protection from the pathological and lethal effects of EMCV infection in *Peli3*$^{-/-}$ mice was associated with a lower viral load in cardiac tissue, as assessed by mRNA expression of the EMCV replicase gene (Fig. 3.22d). Indeed, this was consistent with increased IFN-β expression in *Peli3*$^{-/-}$ mice hearts infected with the virus; which presumably facilitated the increased viral clearance and host protection. The increase in viral clearance was not associated with a stronger pro-inflammatory response as no significant differences in TNF-α mRNA were detectable between WT and *Peli3*$^{-/-}$ mice. Taken together, Pellino3 plays a critical physiological and regulatory role in controlling type 1 IFN production in response to viral infection.

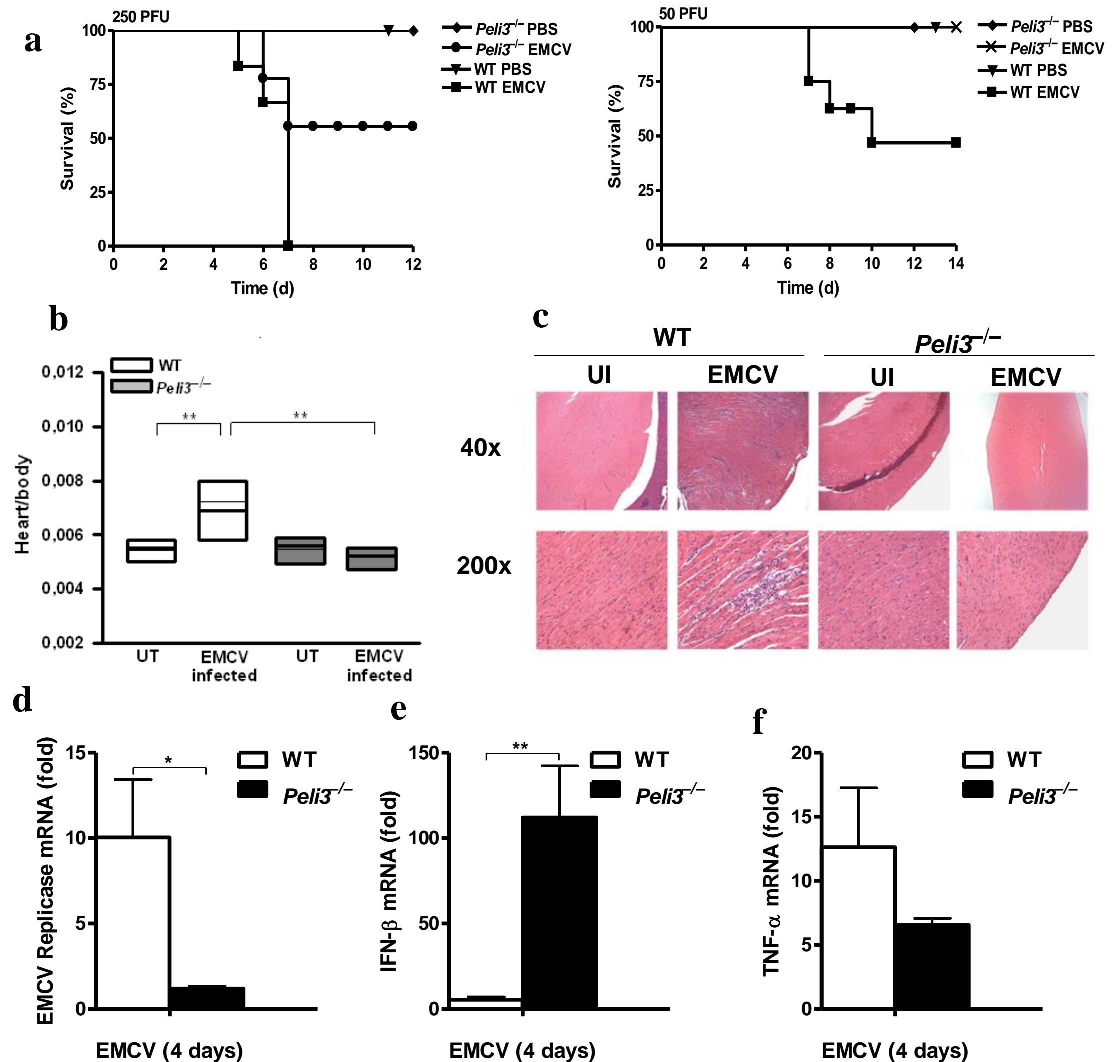

Figure 3.22 Pellino3-deficiency leads to enhanced host protection against EMCV infection.

(**a**) Survival rates of age-matched and sex-matched wild-type (WT; *n* = 7) and *Peli3*$^{-/-}$ (*n* = 7) mice after intraperitoneal injection with 250 PFU or 50 PFU of EMCV virus or with PBS as a vehicle control (*P* = 0.005, Kaplan-Meier analysis). Moribund mice were killed. Surviving mice were killed 12 d or 14 d after infection. **(b)** Heart/body weight ratios **(c)** and heart samples stained with hematoxylin and eosin from WT and *Peli3*$^{-/-}$ mice after intraperitoneal injection with EMCV (50 PFU). UT, untreated. Original magnification, ×40 (top) and ×200 (bottom). Quantitative PCR of mRNA expression for **(d)** EMCV replicase, **(e)** IFN-β and **(f)** TNF-α in heart tissue from WT and *Peli3*$^{-/-}$ mice 4 d after intraperitoneal injection with 50 PFU EMCV (*n* = 3 mice each group). Error bars, S.E.M. (*n* = 3 experiments). *, $p < 0.05$; ** $p < 0.01$ (paired Student's *t*-test). (Data provided by Dr. Jakub Siednieko. Histological analysis conducted by Dr. John J. Callanan. Data adapted from Siednienko, Jackson *et al.,* 2012).

3.2.18 Pellino3 does not Regulate Secondary type 1 IFN-induced Signalling

Studies next progressed to probe the mechanism underlying the negative regulatory effects of Pellino3 on the TLR3 pathway. Given that the initial induction of type 1 IFNs by TLR3 can feed forward to enhance further expression of type 1 IFNs and related genes, it was important to exclude a role for Pellino3 in regulating this secondary induction of type 1 IFN signalling. As type 1 IFN signalling results in the activation of the STAT proteins leading to up regulation of ISGs, the role for Pellino3 in STAT1 and STAT3 activation was conducted. BMDCs from WT and *Peli3*$^{-/-}$ mice were stimulated with recombinant murine IFN-α4 for the indicated times (Fig. 3.23). Protein lysates were generated and probed for phosphorylation of STAT1 and STAT3. Activation was strongly induced after 15 m and 30 m stimulation, with dephosphorylation occurring upon longer exposure to the cytokine stimulus. These phosphorylation kinetics were identical in cells from both the WT and *Peli3*$^{-/-}$ dendritic cells indicating a lack of a role for Pellino3 in this signalling pathway. This highlights that Pellino3's role in the regulation of type 1 IFN expression is likely to be solely restricted to targeting the primary and direct phase of TLR3 signalling.

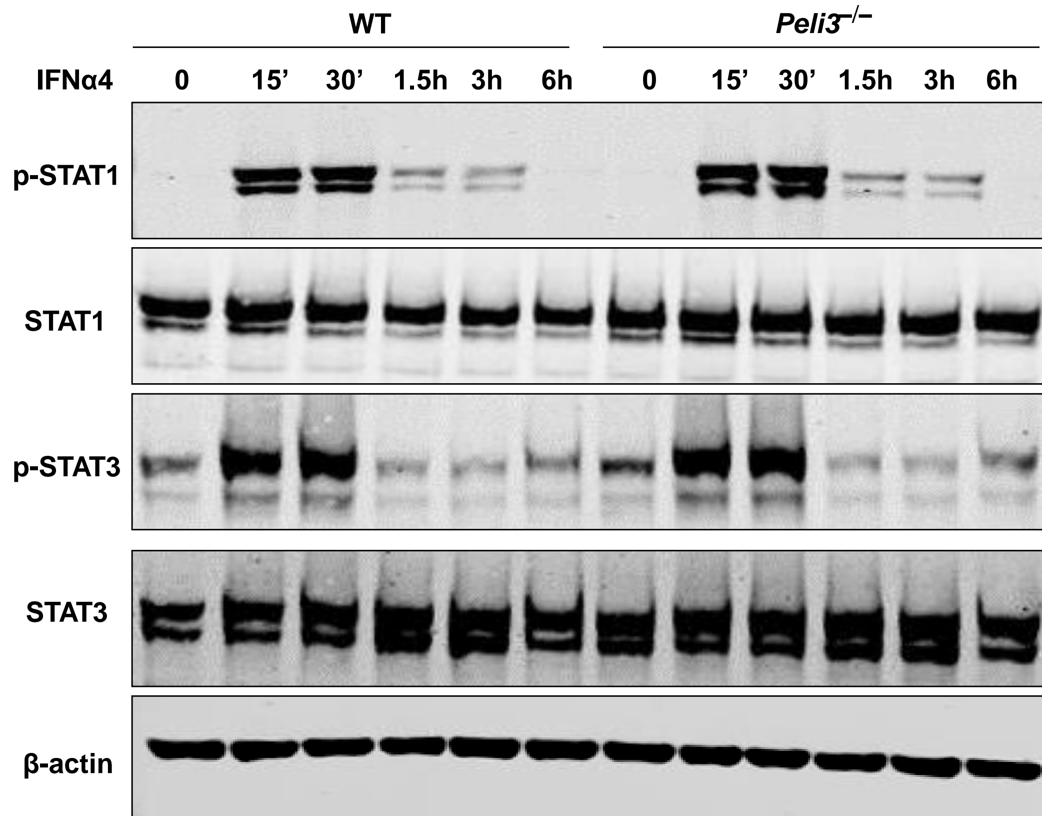

Figure 3.23 Effect of Pellino3-deficiency on IFN-α-induced phosphorylation of STAT1 and STAT3 in BMDCs.

BMDCs were isolated from WT and *Peli3*$^{-/-}$ mice and were left untreated (0) or treated with recombinant murine IFNα4 (1 μg/ml) for the indicated times. Cell lysates were generated and subjected to immunoblot analysis for phosphorylated (p-) STAT1 and p-STAT3. Lysates were also probed for the levels of total STAT1, STAT3 and β-actin to act as a loading control. Data are representative of 2 independent experiments.

3.2.19 Pellino3 does not Contribute to TLR3-induced Activation of MAPKs or AP-1

Previous work on Pellino3 had indicated it may have a role in the MAPK p-38 activation and AP-1-induced transcription factor activity (Jensen and Whitehead, 2003c, Butler et al., 2005) . Given that AP-1 binds the PRDIV of the IFN-β promoter and that TRIF-dependent phosphorylation of p38 leads to the stabilisation of IFN-β mRNA (Johnsen *et al*., 2012, Wathelet *et al*., 1998), the role of Pellino3 in MAPK and AP-1 activation was next explored. BMDMs (Fig. 3.24) and BMDCs (Fig. 3.25) from WT and *Peli3*$^{-/-}$ mice were stimulated with Poly(I:C) for the indicated times. TLR3 activation resulted in increased phosphorylation of p38, ERK and JNK at 30 min and 45 min post stimulation. Interestingly however, the activated MAPK levels showed comparable levels in both WT and Pellino3-deficent cells. Since activation of MAPKs also results in phosphorylation the of transcription factor c-JUN, a member of the AP-1 family, its phosphorylation status was next investigated. Phosphorylation of c-Jun occurred after 45 m stimulation and was maintained until 3 h post activation. Once again however, no differences in c-JUN activation were observed in the absence of Pellino3. These findings indicate that any regulatory effects of Pellino3 in the TLR3 pathway are not mediated by targeting the activation of MAPKs or AP-1.

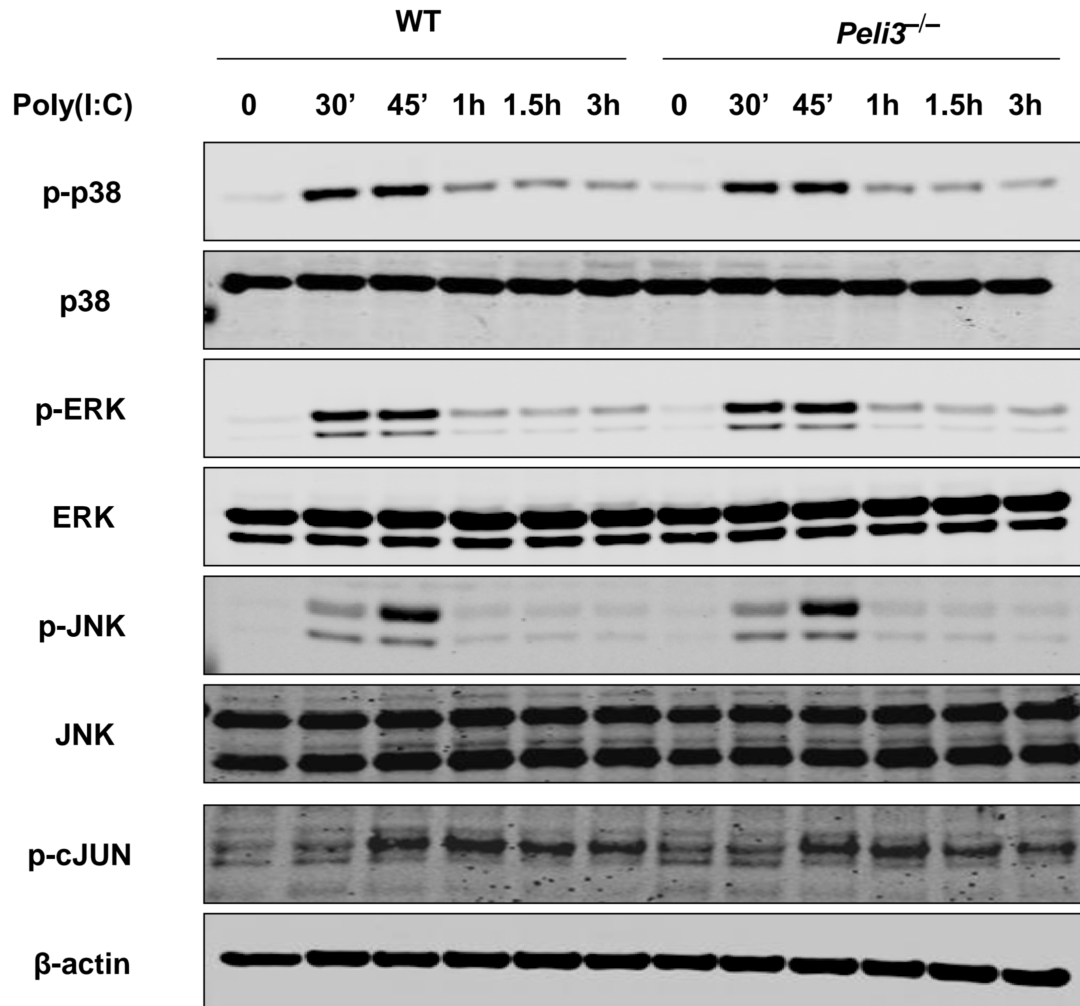

Figure 3.24 Effect of Pellino3-deficiency on TLR3-induced activation of MAPK and AP-1 pathways in BMDMs.

BMDMs were isolated from WT and *Peli3*$^{-/-}$ mice and were left untreated (0) or treated with Poly(I:C) (25 μg/ml) for the indicated times. Cell lysates were generated and subjected to immunoblot analysis for p-p38, p-ERK and p-JNK. Lysates were also probed for the levels of total p38, ERK, JNK and β-actin to act as a loading control. Data are a representative of 3 independent experiments.

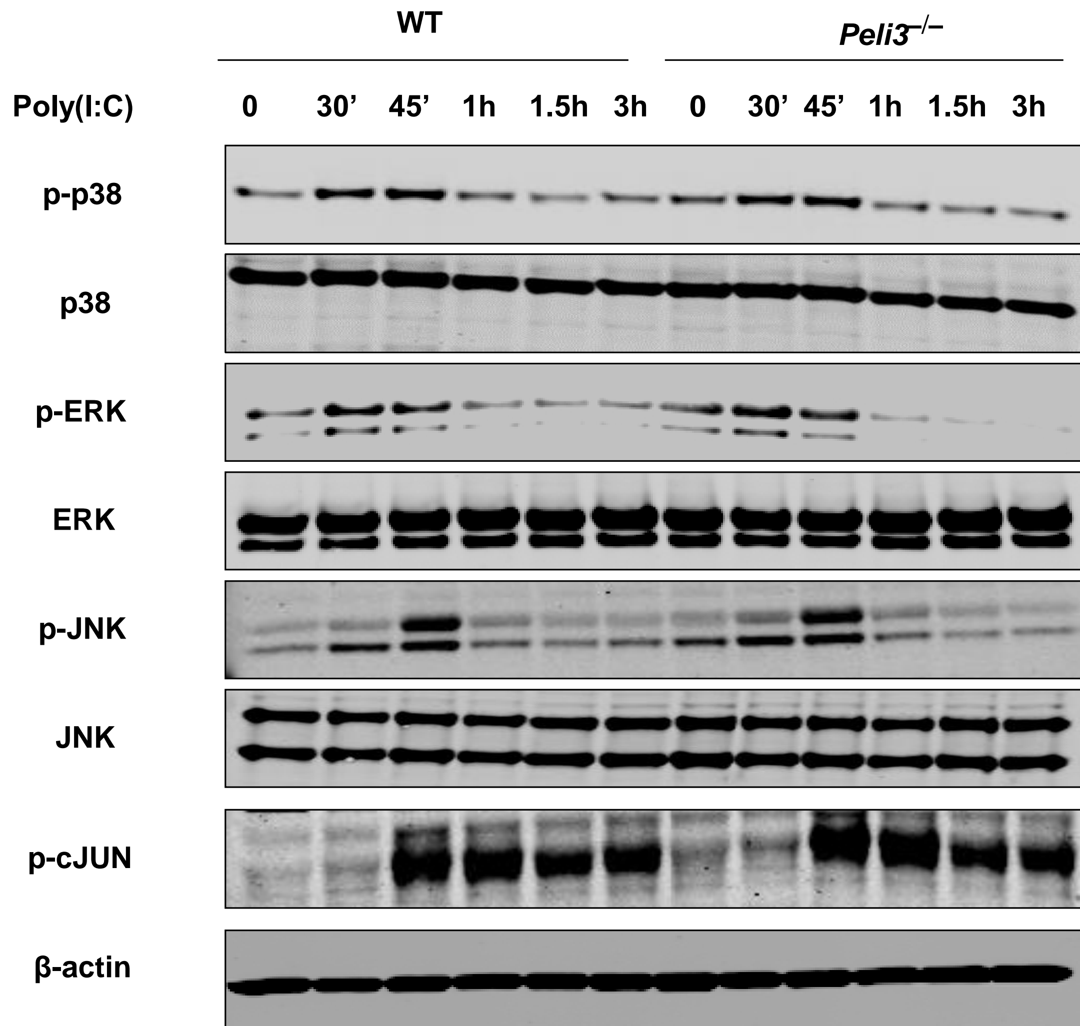

Figure 3.25 Effect of Pellino3-deficiency on TLR3-induced activation of MAPK and AP-1 pathways in BMDCs.

BMDCs were isolated from WT and *Peli3*$^{-/-}$ mice and were left untreated (0) or treated with Poly(I:C) (25 μg/ml) for the indicated times. Cell lysates were generated and subjected to immunoblot analysis for p-p38, p-ERK and p-JNK. Lysates were also probed for the levels of total p38, ERK, JNK and β-actin to act as a loading control. Data are a representative of 3 independent experiments.

3.2.20 NF-κB activation is not Regulated by Pellino3 during TLR3 Signalling

TLR3-induced expression of type 1 IFNs requires the coordinated activation of a number of transcription factors. The NF-κB family of transcription factors have been shown to bind to the PRDII of the IFN-β promoter and induce cytokine transcription in response to viral stimuli (Mesplede *et al*., 2003). The canonical NF-κB pathway was next assessed as a potential target for the regulatory effects of Pellino3. As activation of IKKs lead to the phosphorylation and subsequent degradation of the NF-κB cytosolic sequestering protein IκBα (Zandi *et al*., 1998), their phosphorylation kinetics were investigated as an index of NF-κB activation. BMDMs (Fig. 3.26) and BMDCs (Fig. 3.27) were stimulated with Poly(I:C) for indicated times. The absence of Pellino3 did not affect the capacity of Poly(I:C) to induced the phosphorylation of the IKKs. Robust phosphorylation of IKKα/β can be seen in both WT and Pellino3-deficent cells 30 min post stimulation, with phosphorylated kinase still evident at 3 h. Phosphorylation of their downstream substrate IκBα is also unaffected by Pellino3-deficency. After IκBα phosphorylation it is known to be K48-linked polyubiquitinated and targeted for 26S proteasomal degradation (Alkalay *et al*., 1995). This is demonstrated by the decrease in total IκBα protein abundance seen after 30 m stimulation. However, both the degradation and re-synthesis of IκBα is again unaffected in Pellino3-deficent cells. Recently, the NF-κB subunit p65 has been shown to play a vital role in viral-induced IFN-β production (Bartlett *et al*., 2012), however, although phosphorylation of p65 at serine 536 is strongly induced by TLR3 activation there is no phenotypic difference between WT and Pellino3-deficent cells. Whilst Pellino3 fails to regulate upstream regulatory pathways that control NF-κB, it was important to assess if it could affect the binding of NF-κB to its DNA recognition motif. Nuclear extracts were generated from BMDCs from WT and *Peli3*$^{-/-}$ mice, previously stimulated with Poly(I:C) and subjected to EMSA analysis. In WT cells Poly(I:C)-induced a time dependant increase in the amount of nuclear NF-κB that could bind with a labelled NF-κB consensus site oligonucleotide (Fig. 3.28). This pattern was also observed in Pellino3-deficient cells indicating that Pellino3 does not target NF-κB in the TLR3

pathway. In order to ensure specificity of the NF-κB-oligonucleotide binding reaction, a non-labelled form of the same oligonucleotide successfully competed with the labelled oligonucleotide for binding to the nuclear extracts.

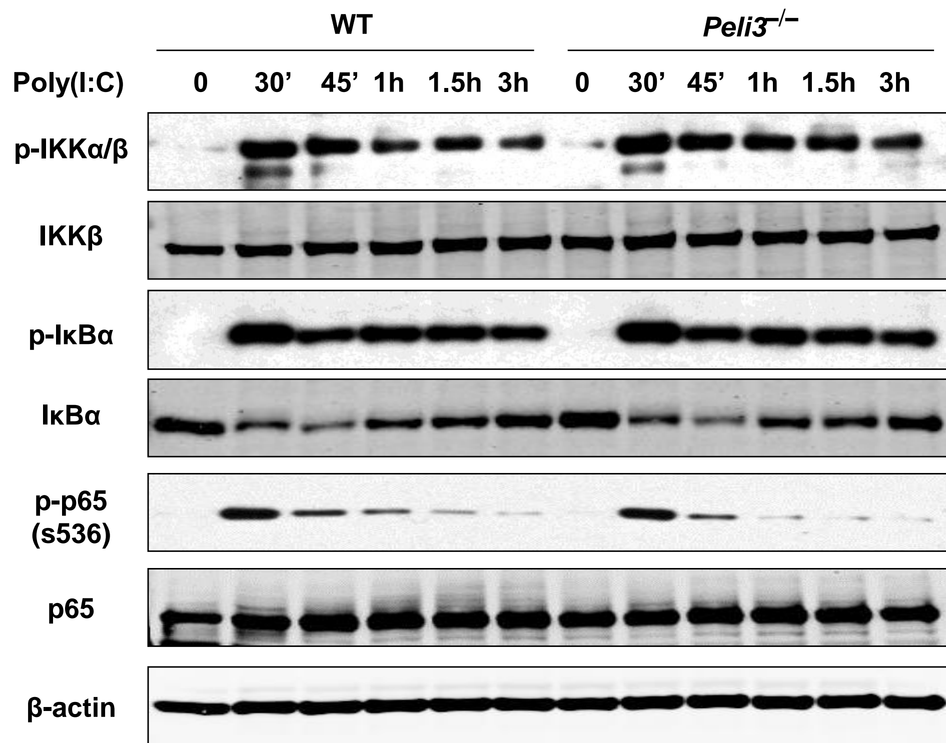

Figure 3.26 Effect of Pellino3-deficiency on TLR3-induced activation of the NF-κB pathway in BMDMs.

BMDMs were isolated from WT and *Peli3*$^{-/-}$ mice and were left untreated (0) or treated with Poly(I:C) (25 μg/ml) for the indicated times. Cell lysates were generated and subjected to immunoblot analysis for p-IKKα/β, p-IκBα and p-p65. Lysates were also probed for the levels of total IKKβ, IκBα, p65 and β-actin to act as a loading control. Data are a representative of 3 independent experiments.

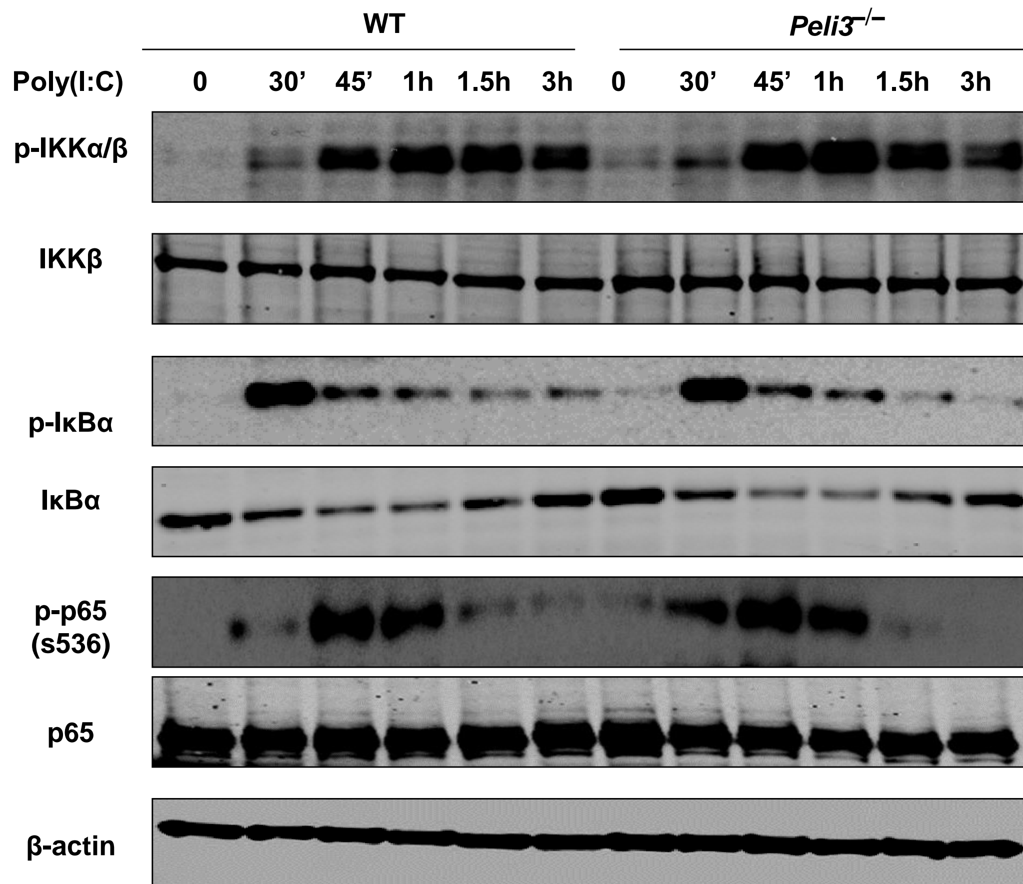

Figure 3.27 Effect of Pellino3-deficiency on TLR3-induced activation of the NF-κB pathway in BMDCs.

BMDCs were isolated from WT and *Peli3*$^{-/-}$ mice and were left untreated (0') or treated with Poly(I:C) (25 μg/ml) for the indicated times. Cell lysates were generated and subjected to immunoblot analysis for p-IKKα/β, p-IκBα and p-p65. Lysates were also probed for the levels of total IKKβ, IκBα, p65 and β-actin to act as a loading control. Data are a representative of 3 independent experiments.

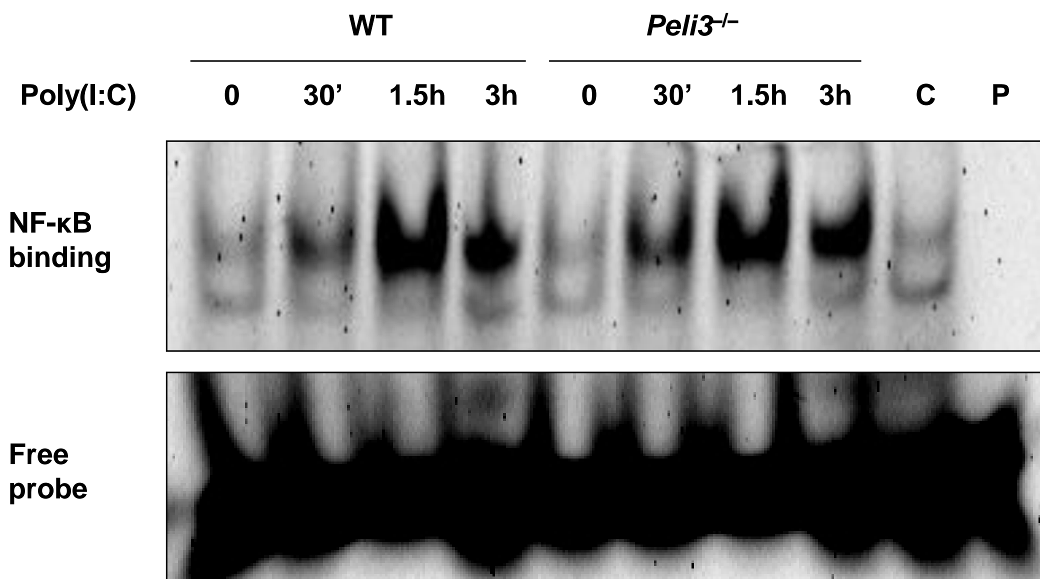

Figure 3.28 Effect of Pellino3-deficiency on TLR3-induced binding of NF-κB to DNA

BMDCs were isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (0) or treated with Poly (I:C) (25 µg/ml) for the indicated times. Nuclear extracts (5 µg protein) were incubated with an IR-labelled oligonucleotide containing the NF-κB-binding consensus site and subjected to native PAGE. The specificity of the NF-κB-DNA binding reaction was confirmed by the absence of the complex when the nuclear extract from WT BMDCs (treated with Poly (I:C) for 90 min) was pre-incubated with unlabelled competitor (C) oligonucleotide. The labelled oligonucleotide probe (P) was also run alone as a negative control. Data are a representative of 3 independent experiments.

3.2.21 Pellino3 does not Affect TBK1 or IRF3 Activation by TLR3

Another critical component in type 1 IFN production by TLR3 is the activation of the IRF transcription factors. IRF3 and its upstream kinase TBK1 are absolutely required for type 1 IFN and related gene expression, as deficiency in either lead to signal abrogation during TLR3 signalling (McWhirter et al., 2004a). Therefore, their phosphorylation kinetics was next examined in response to Poly(I;C). BMDMs (Fig. 3.29) and BMDCs (Fig. 3.30) from WT and Pellino3-deficent mice were stimulated with Poly(I:C) for the indicated times and protein lysates generated. Western blot analysis of WT cells revealed that Poly(I:C)-induced time dependent phosphorylation of TBK1 after 30 min stimulation displaying some reduction by 3 h. However, this pattern was also observed in Pellino3-deficient cells excluding TBK1 as a target for Pellino3. TBK1 is well known to phosphorylate and activate IRF3. So to investigate if Pellino3 may function downstream of TBK1, Poly(I:C)-induced phosphorylation of IRF3 phosphorylation was next explored. After 45 min Poly(I:C) stimulation, BMDMs and BMDCs from WT and Pellino3-deficent mice exhibited comparable levels of IRF3 phosphorylation. Phosphorylated levels peaked between 1 h and 1.5 h and were returning to basal levels by 3 h. These data indicated that Pellino3 does not target the activation of IRF3 in the TLR3 pathway.

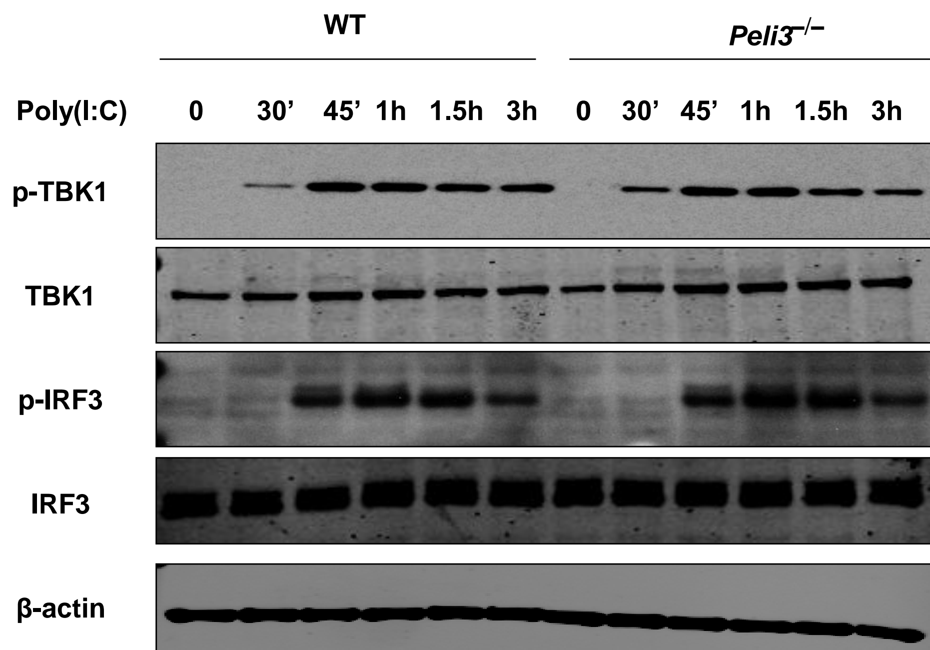

Figure 3.29 Effect of Pellino3-deficiency on TLR3-induced activation of the IRF3 pathway in BMDMs.

BMDMs were isolated from WT and *Peli3*$^{-/-}$ mice and were left untreated (0) or treated with Poly(I:C) (25 µg/ml) for the indicated time. Cell lysates were generated and subjected to immunoblot analysis for p-TBK1 and p-IRF3. Lysates were also probed for the levels of total TBK1, IRF3 and β-actin to act as a loading control. Data are a representative of 3 independent experiments.

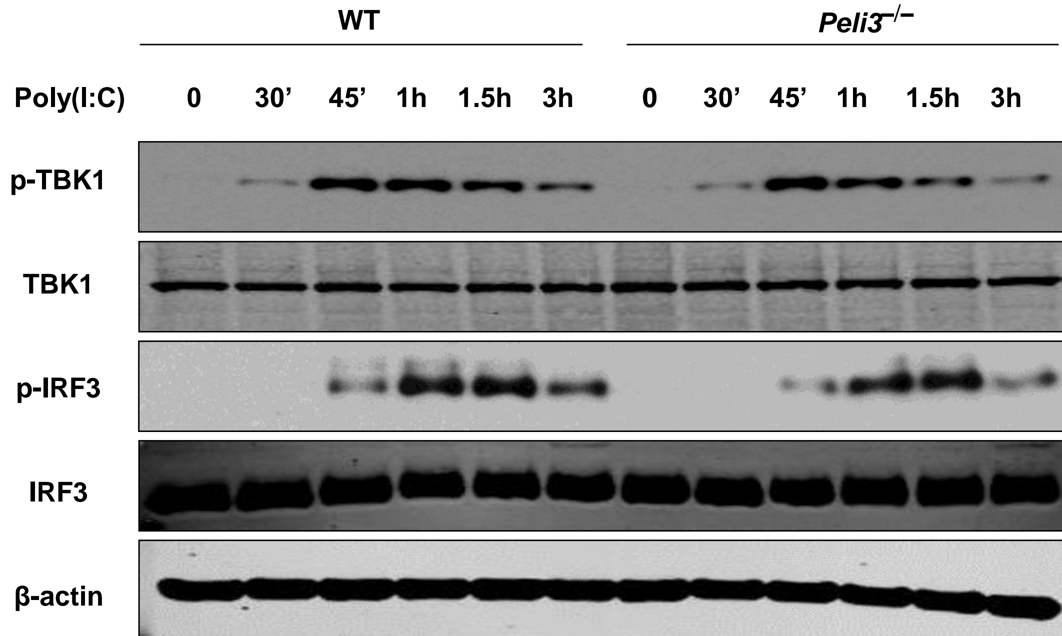

Figure 3.30 Effect of Pellino3-deficiency on TLR3-induced activation of the IRF3 pathway in BMDCs.

BMDCs were isolated from WT and *Peli3*$^{-/-}$ mice and were left untreated (0) or treated with Poly(I:C) (25 μg/ml) for the indicated time. Cell lysates were generated and subjected to immunoblot analysis for p-TBK1 and p-IRF3. Lysates were also probed for the levels of total TBK1, IRF3 and β-actin to act as a loading control. Data are a representative of 3 independent experiments.

3.2.22 Pellino3 Negatively Regulates TLR3-induced Binding of Proteins to the PRDI/III Region of the IFN-β Promoter

Although IRF3 activation was not affected by Pellino3-deficiency, it was possible that Pellino3 may be targeting IRF7. The PRDI/III site of the IFN-β promoter is recognised by both IRF3 and IRF7 and is critically important in the upregulation of the anti-viral cytokine and so we next used EMSA analysis to assess the effects of Pellino3-deficiency on TLR3-induced binding of proteins to the PRDI/III motif. MEFs were isolated from WT and *Peli3*$^{-/-}$ mice and stimulated with Poly(I:C) for the indicated times and nuclear fractions generated. Incubation of the nuclear fraction from Poly(I:C)-treated WT cells with a labelled oligonucleotide containing the PRDI/III motif from the IFN-β promoter revealed modest DNA binding in extracts after 3 h stimulation (Fig. 3.31). Interestingly, DNA-protein interaction was evident earlier at 1.5 h in the Pellino3-deficient cells, with further augmentation seen at 3 h compared to the WT cells. In order to ensure specificity of the reaction, a non-labelled oligonucleotide was pre-incubated for 15 m with the *Peli3*$^{-/-}$ 3 h stimulated nuclear fraction prior to labelled oligonucleotide incubation. The absence of detected DNA protein binding in this competitor (C) sample is indicative of a specific PRD I/III DNA interaction. This increase in IRF PRDI/III association seen in cells isolated from *Peli3*$^{-/-}$ mice suggested that Pellino3 was somehow targeting the IRF binding to the PRDI/III domain of the IFNB promoter.

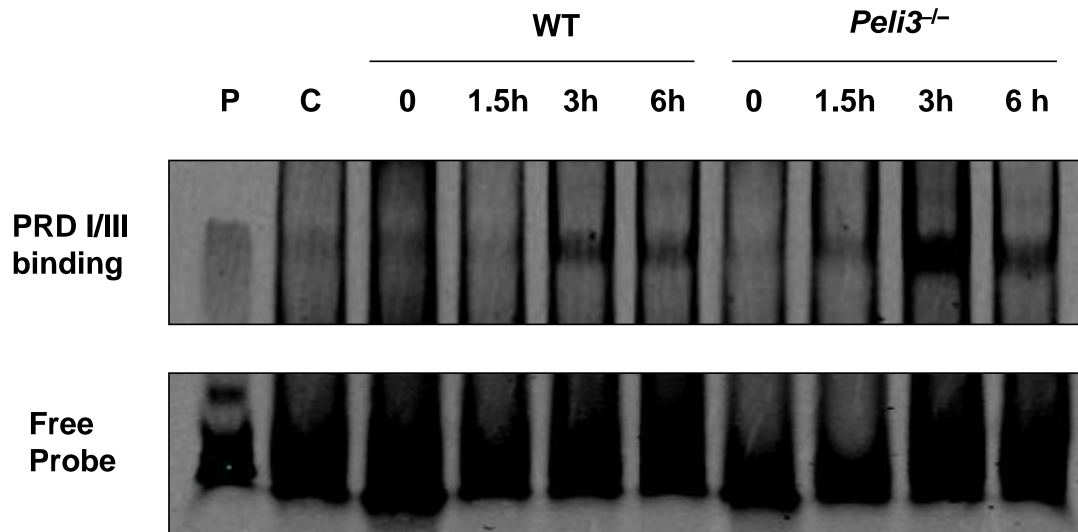

Figure 3.31 Effect of Pellino3-deficiency on TLR3-induced binding of IRF3/7 to DNA

MEFs were isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (0) or treated with Poly (I:C) (25 µg/ml) for the indicated times. Nuclear extracts (5 µg protein) were incubated with an IR-labelled oligonucleotide containing the IRF3/7-binding consensus site and subjected to native PAGE. The specificity of the IRF-DNA binding reaction was confirmed by the absence of the complex when the nuclear extract from *Peli3*$^{-/-}$ cells (treated with Poly (I:C) for 3 h) was pre-incubated with unlabelled competitor (C) oligonucleotide. The labelled oligonucleotide probe (P) was also run alone as a negative control. Data are a representative of 3 independent experiments.

3.2.23 Pellino3 Negatively Regulates Poly(I:C)-induced Phosphorylation of IRF7

Given that IRF3 and IRF7 can both bind to the PRDI/III region of IFN-β promoter, and Pellino3-deficiency does not affect IRF3 activation, the regulatory effect of Pellino3 on IRF7 was next examined. Unfortunately, no commercially available phospho-specific antibody for IRF7 is available to detect endogenous phosphorylated-IRF7 and so an indirect approach was used in which IRF7 was immunoprecipitated and probed for phosphorylation using an anti-phospho-serine antibody. BMDCs from WT and *Peli3*$^{-/-}$ mice were stimulated with Poly(I:C) for the indicated times (Fig. 3.32). In order to dissociate any protein-protein interactions, cell lysates were generated and 10% SDS added followed by sample boiling for 5 min. The SDS was diluted to 1% and endogenous IRF7 was then immunoprecipitated using an anti-IRF7 antibody. The enriched lysate which should only contain IRF7 and its covalent modifications and lysates were probed for the presence of phosphorylated serine residues. Poly(I:C)-induced a modest time dependent increase in IRF7 phosphorylation in WT cells which was substantially higher in the absence of Pellino3. Although IRF7 protein levels can be upregulated by type 1 IFN signalling, immunoblotting for total IRF7 revealed no difference in the protein abundance in this experimental model. This suggested that Pellino3 may regulate the phosphorylation of IRF7.

3.2.24 Pellino3 Specifically Inhibits IRF7 Nuclear Translocation

As phosphorylation of IRF7 is a prerequisite for its nuclear translocation, the ability of Pellino3 to regulate this process was next examined. Nuclear fractions were isolated from BMDCs from WT and *Peli3*$^{-/-}$ mice after Poly(I:C) stimulation for the indicated durations (Fig. 3.33). In both WT and Pellino3-deficent cells, Poly(I:C)-induced a time dependent nuclear translocation of IRF7 however, the levels of nuclear IRF7 were further augmented in the Pellino3-deficent cells compared to the

WT controls. In order to confirm Pellino3 primarily targeted IRF7 and not IRF3, IRF3's translocation was also examined. However, the Poly(I:C)-induced nuclear localisation of IRF3 was comparable in both WT and *Peli3*$^{-/-}$ cells suggesting that Pellino3 specifically targets IRF7 and not IRF3 in the TLR3 pathway.

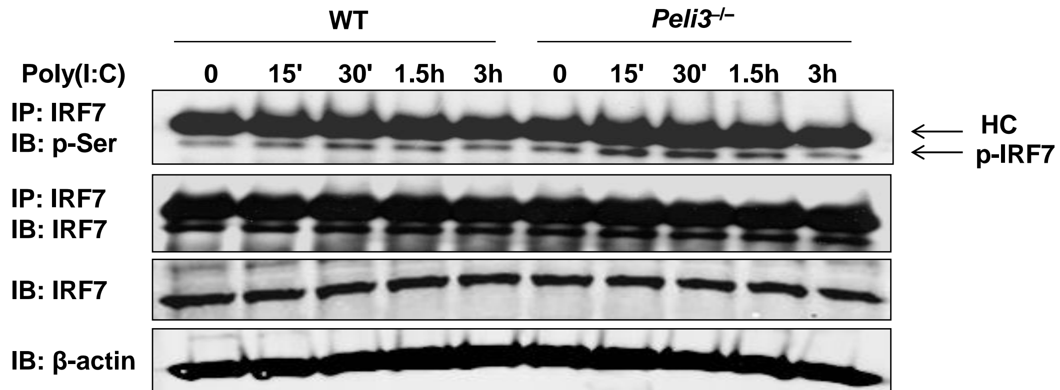

Figure 3.32 Effect of Pellino3-deficiency on TLR3-induced phosphorylation of IRF7 in BMDCs.

BMDCs were isolated from WT and *Peli3*$^{-/-}$ mice and were left untreated (0) or treated with Poly(I:C) (10 μg/ml) for the indicated times. Cell lysates were generated and subjected to immunoprecipitation (IP) with an anti-IRF7 antibody and immunoblotted (IB) for phosphorylated serine residues (p-Ser) on IRF7. Immunoprecipitated samples and whole cell lysates were also immunoblotted for total IRF7 and β-actin to act as a loading control. The arrows indicate the heavy chain (HC) of the IP antibody and the serine phosphorylated form of IRF7 (p-IRF7). Data are a representative of 3 independent experiments.

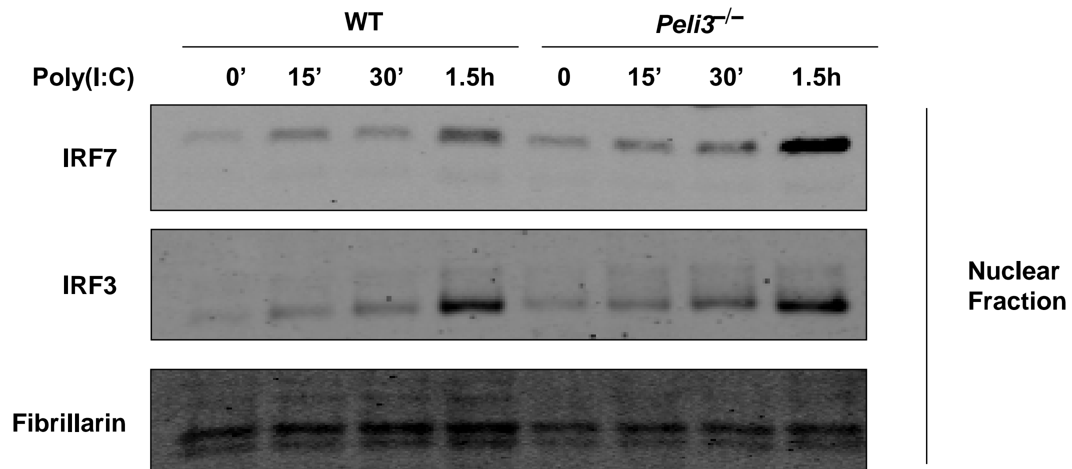

Figure 3.33 Effect of Pellino3-deficiency on TLR3-induced nuclear translocation of IRF3 and IRF7.

BMDCs were isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (0) or treated with Poly(I:C) (25 μg/ml) for the indicated times. Nuclear fractions were subjected to immunoblotting using antibodies against IRF7, IRF3 and Fibrillarin (loading control). Data are representative of 3 individual experiments.

3.2.25 Pellino3 Negatively Regulates TLR3-induced Polyubiquitination of IRF7

The mechanism by which Pellino3 was targeting the IRF7 pathway was next explored. As loss of Pellino3 had no effect on the upstream kinase for IRF7 and Pellino3 is itself an E3 ligase, the polyubiquitination of IRF7 was investigated in Pellino3-deficient cells. In order to examine this modification, the same immunoprecipitation approach as described in 3.2.20 was employed but probed for ubiquitination. MEFs (Fig. 3.34a) and BMDCs (Fig. 3.34b) isolated from WT and *Peli3*$^{-/-}$ mice were stimulated with Poly(I:C) for the indicated times. In MEFs from WT mice, Poly(I:C)-induced a ubiquitination of IRF7 after 30 m stimulation. This was further augmented and sustained in cells from *Peli3*$^{-/-}$ mice. In addition, the ubiquitinated IRF7 appears more abundant in the Pellino3-deficient cells at all time points. In BMDCs, again IRF7 polyubiquitination occurs earlier in cells from Pellino3-deficent mice and the intensity of the ubiquitination appears greater than what is observed in cells from WT mice after Poly(I:C) treatment. It has previously been reported that IRF7 polyubiquitination is a prerequisite for activation of IRF7 (Ning *et al.*, 2008a), indicating that this increased ubiquitination of IRF7 in Pellino3-deficient cells may facilitate the augmented responses to Poly(I:C) and EMCV in *Peli3*$^{-/-}$ mice.

3.2.26 Pellino3 Inhibits TLR3-induced TRAF6-IRF7 Interaction

As there is increased polyubiquitination of IRF7 in the absence of Pellino3, investigation into the E3 ligase responsible was conducted. As TLR7/8 signalling in pDC results in TRAF6-mediated activation of IRF7 (Kawai *et al.*, 2004) and TRAF6 has been shown to directly interact with and ubiquitinate IRF7 (Ning *et al.*, 2008a), the effects of Pellino3-deficiency on TRAF6-IRF7 interaction was next explored. BMDCs were isolated from WT and *Peli3*$^{-/-}$ mice and stimulated with Poly(I:C) for the indicated times. Cell lysates were generated and samples were

immunoprecipitated with an antibody for IRF7. Samples were then probed for the presence of co-precipitating TRAF6 protein. TLR3 activation induced interaction between IRF7 and TRAF6 in a time-dependent manner in BMDCs from WT mice (Fig. 3.35). Interestingly, this interaction was increased in the absence of Pellino3 suggesting that Pellino3 regulates the ubiquitination of IRF7 by modulating the interaction of IRF7 with its E3 ubiquitin ligase TRAF6.

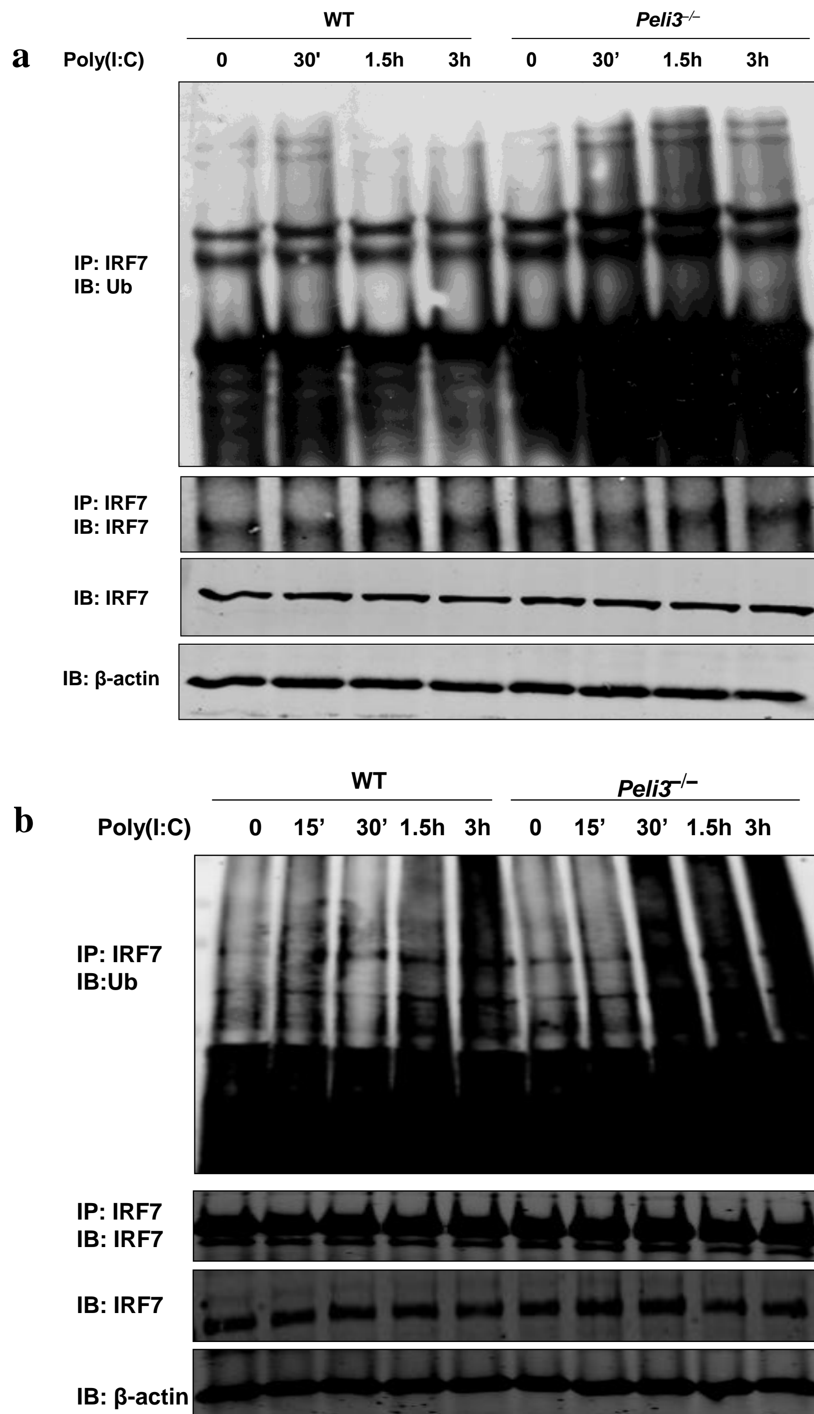

Figure 3.34 Effect of Pellino3-deficiency on TLR3-induced IRF7 polyubiquitination.

(a) MEFs and **(b)** BMDCs were isolated from WT and *Peli3*$^{-/-}$ were left untreated (0) or treated with Poly(I:C) ((**a**) 25 µg/ml or **(b)** 10 µg/ml) for the indicated time. Cell lysates were generated and subjected to immunoprecipitation (IP) with an anti-IRF7 antibody and immunoblotted (IB) for polyubiquitinated IRF7. Immunoprecipitated samples and whole cell lysates were also immunoblotted for total IRF7 and β-actin to act as a loading control. Data are a representative of 3 independent experiments.

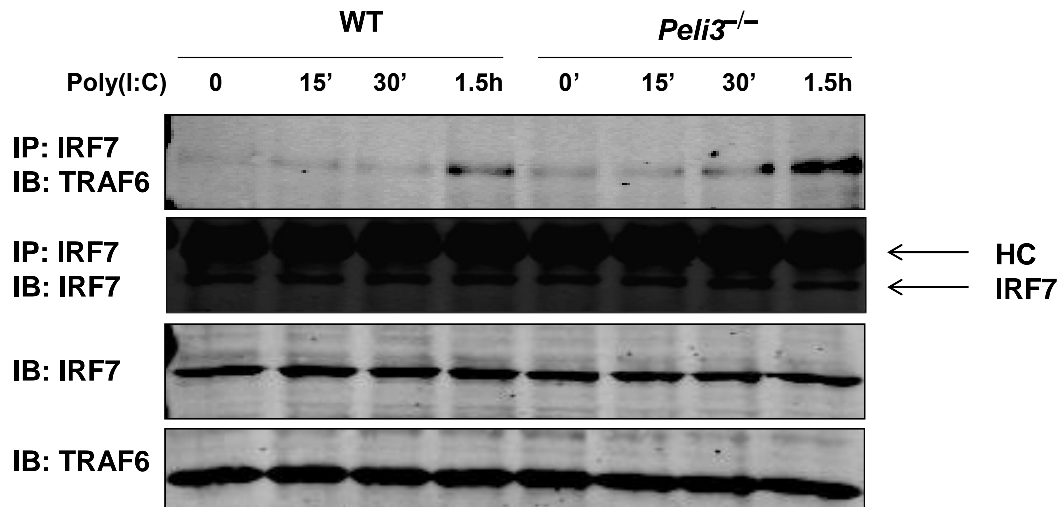

Figure 3.35 Effect of Pellino3-deficiency on TLR3-induced TRAF6 and IRF7 interaction

BMDCs were isolated from WT and *Peli3*$^{-/-}$ were left untreated (0') or treated with Poly(I:C) (10 µg/ml) for the indicated time. Cell lysates were generated and subjected to immunoprecipitation (IP) with an anti-IRF7 antibody and immunobloted IB) for TRAF6. Immunoprecipitated samples and whole cell lysates were also probed for the levels of total IRF7 and β-actin to act as a loading control. The arrows indicate the heavy chain (HC) of the IP antibody and the total IRF7 protein. Data is a representative of 3 independent experiments.

3.2.27 TRAF6 Mediates TLR3-induced Cytokine Production

The data presented above suggests that TRAF6-mediated IRF7 polyubiquitination may be an important target for Pellino3 and for manifesting its regulatory effects on the TLR3 pathway. However, such a model would require demonstration of an important mediatory role for TRAF6 and its E3 ligase activity in the TLR3 pathway that regulates type 1 IFN expression. MEFs from WT, *Peli3*$^{-/-}$**,** *Traf6*$^{-/-}$ mice were thus stimulated with Poly(I:C) for 6 h and mRNA extracted. Real-time quantitative PCR for IFN-β confirmed, as before, that Pellino3 negatively regulates TLR3-induced IFN-β (Fig. 3.36a). Interestingly, a complete loss of TLR3-induced IFN-β mRNA expression was exhibited in the *Traf6*$^{-/-}$ MEFs highlighting an indispensable role for TRAF6 in TLR3 signalling. In order to confirm previous reports that TRAF6 is essential for TLR3-induced pro-inflammatory cytokine production by the NF-κB pathway in MEFs, real time quantitative PCR for IL-6 was also conducted (Fig. 3.36b). Investigation of *Traf6*$^{-/-}$ in TLR3 activation of IL-6 mRNA expression, confirmed previous studies that implicate TRAF6 as an essential mediator of TLR3 activation of the NF-κB pathway in MEFs (Fig. 3.36b). As seen in BMDMs and BMDCs previously (Fig. 3.6), Pellino3-deficiency had no effect on Poly(I:C)-induced IL-6 transcription in MEFs. Taken together, these results indicate that in MEFs, TRAF6 plays a key role in both pro-inflammatory and anti-viral signalling pathways in response to dsRNA.

3.2.28 TRAF6-E3 Ligase Activity is essential for TLR3 Signal Transduction

We next assessed if the E3 ligase activity of TRAF6 is critically important in TLR3-induced cytokine expression. In order to investigate this, *Traf6*$^{-/-}$ MEFs were reconstituted with 3 μg of DNA encoding the expression constructs for WT TRAF6 or a point mutant in the RING domain of TRAF6$^{(C70A)}$ which abolishes its ability to conjugate polyubiquitin chains. As a control, *Traf6*$^{-/-}$ MEFs were also transfected

with 3 μg of non-protein coding DNA. Reconstituted cells were stimulated with Poly(I:C) for 6 h and mRNA extracted. Real time PCR analysis for IFN-β showed that the WT TRAF6 construct could rescue TLR3-induced IFN-β whereas the point mutant failed to (Fig. 3.37a). In addition, real-time PCR analysis for IL-6 revealed a similar requirement for the E3 ligase functionality of TRAF6 (Fig. 3.37b). These results are consistent with a TLR3 signalling model in which TRAF6-induces ubiquitination of IRF7 thus promoting IFN-β expression.

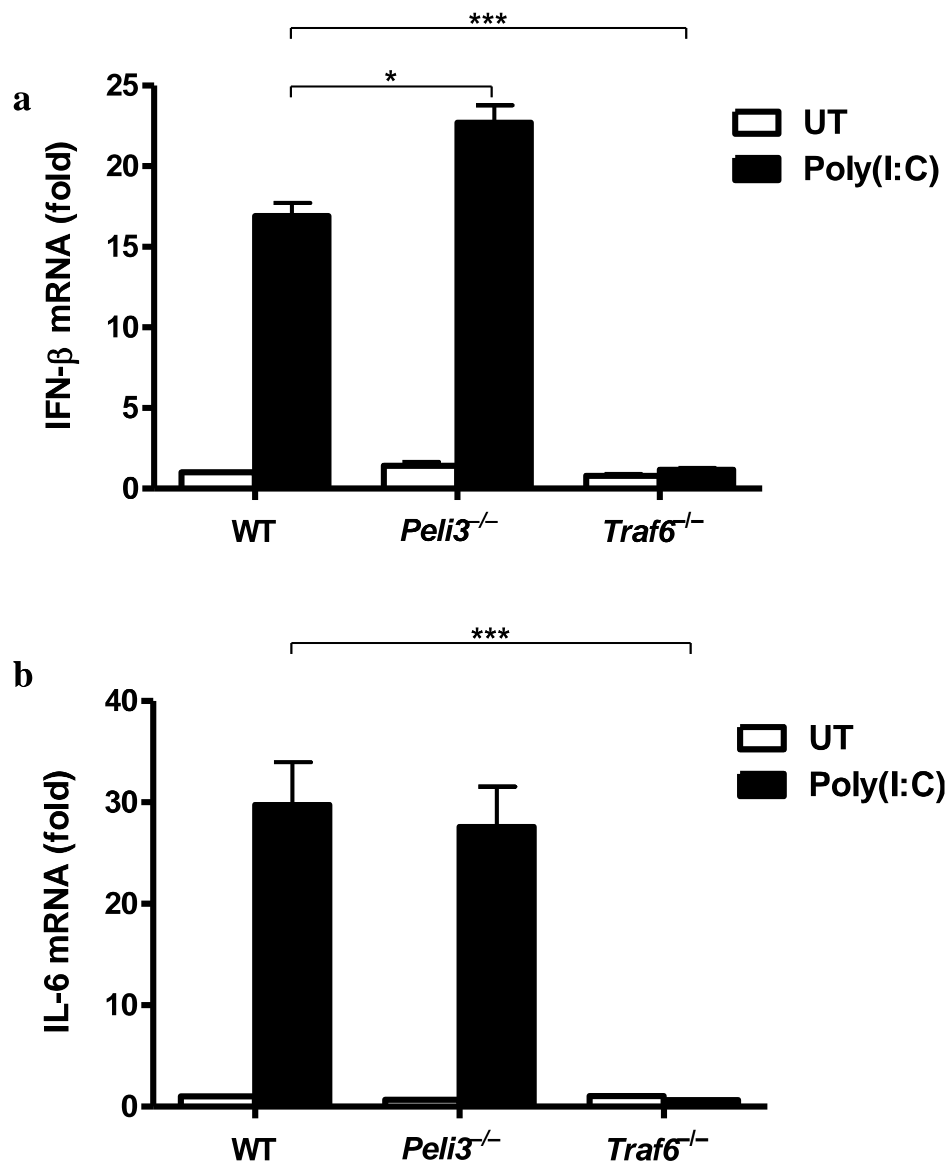

Figure 3.36 Differential effects of Pellino3- and TRAF6-deficiency on TLR3-induced cytokine production in MEFs.

Quantitative real-time PCR of (**a**) IFN-β and (**b**) IL-6 expression in cells from MEFs isolated from WT , *Peli3*$^{-/-}$ and *Traf6*$^{-/-}$ mice and left untreated (UT) or stimulated with Poly(I:C) (25 μg/ml) for 6 h. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$; ***, $p < 0.001$.

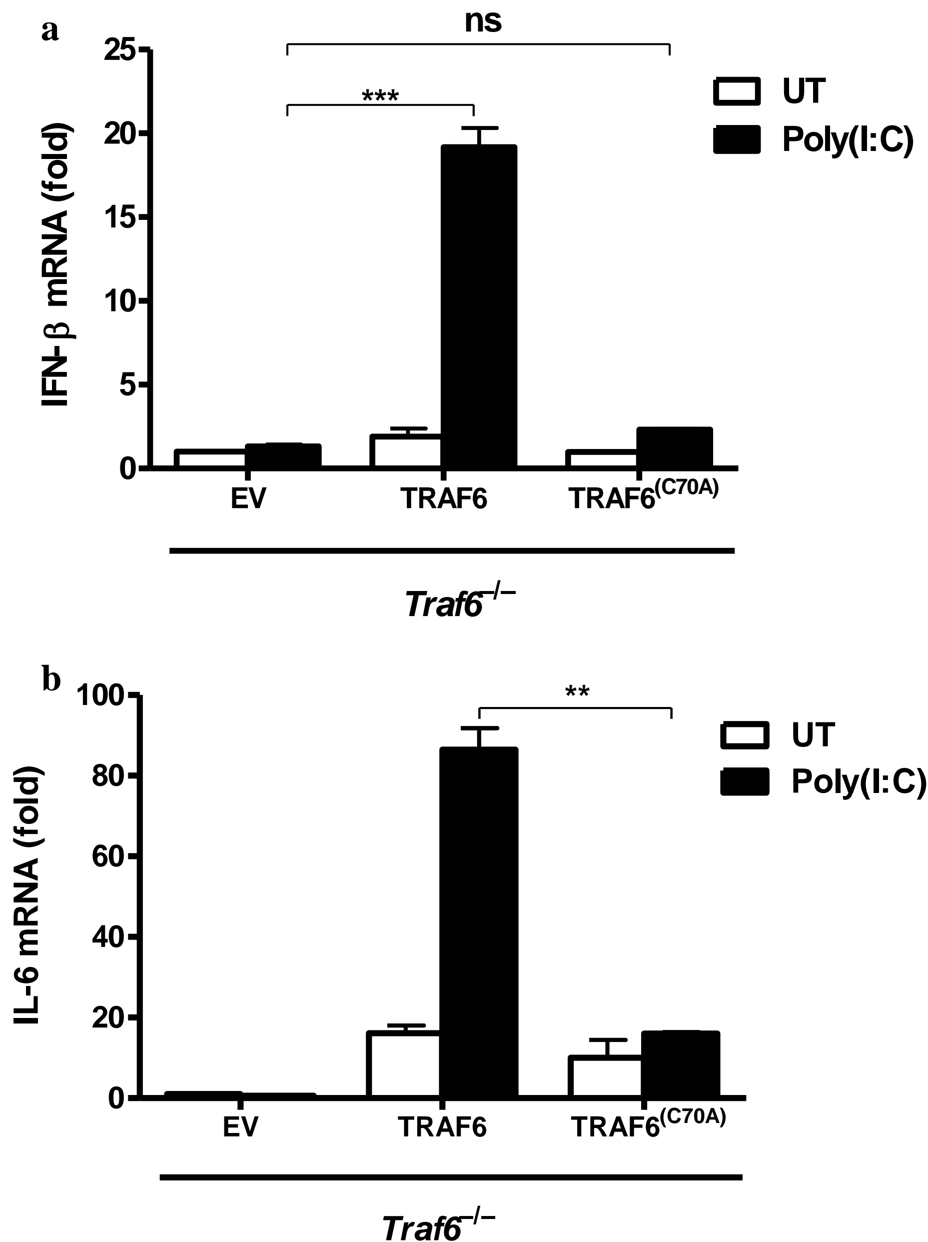

Figure 3.37 TLR3 induction of cytokines is dependent on the E3 ligase activity of TRAF6 in MEFs.

Traf6$^{-/-}$ MEFS were transfected with either an empty vector (EV) or an expression construct encoding WT TRAF6 (TRAF6) or a RING domain mutant (TRAF6$^{(C70A)}$). Quantitative real-time PCR analysis of **(a)** IFN-β and **(b)** IL-6 mRNA expression in untreated (UT) cells or cells stimulated with Poly(I:C) (25 μg/ml) for 6 h. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. **, $p < 0.01$; ***, $p < 0.001$, ns, not significant.

3.2.29 Pellino3 Interacts with TRAF6 during TLR3 Signalling

Given that Pellino3 can regulate the interaction of TRAF6 with IRF7 and subsequent ubiquitination, the ability of Pellino3 to target TRAF6 was next assessed. Initially, HEKT cells stably expressing TLR3 on their surface were transfected with 1 μg of plasmid DNA encoding a non-protein coding empty vector (EV), and/or expression constructs encoding Myc-tagged Pellino3 and/or FLAG-tagged TRAF6. Cell lysates were then split and co-immunoprecipitated with either an anti-Myc or an anti-FLAG antibody. Reciprocal co-interactions studies demonstrated that Pellino3 and TRAF6 could indeed interact when both proteins are overexpressed (Fig. 3.38a). However, although overexpression studies play a fundamental role in identify interacting proteins, they do not indicate if interactions are physiologically relevant at endogenous concentrations or during specific signalling events. In order to address this shortfall, the human derived astrocytoma cell line U373, which naturally expresses TLR3, was stimulated with Poly(I:C) for various times and subjected to co-immunopreciptiation analysis to characterise the interaction of endogenous forms of Pellino3 and TRAF6. Co-immunoprecipitation using an anti-TRAF6 antibody and immunoblotting for Pellino3 revealed a stimulus and time dependent interaction of the proteins peaking at 30 min to 1.5 h which diminishes by 3 h activation (Fig. 3.38b). Therefore, Pellino3 interacts with TRAF6 during TLR3 signalling.

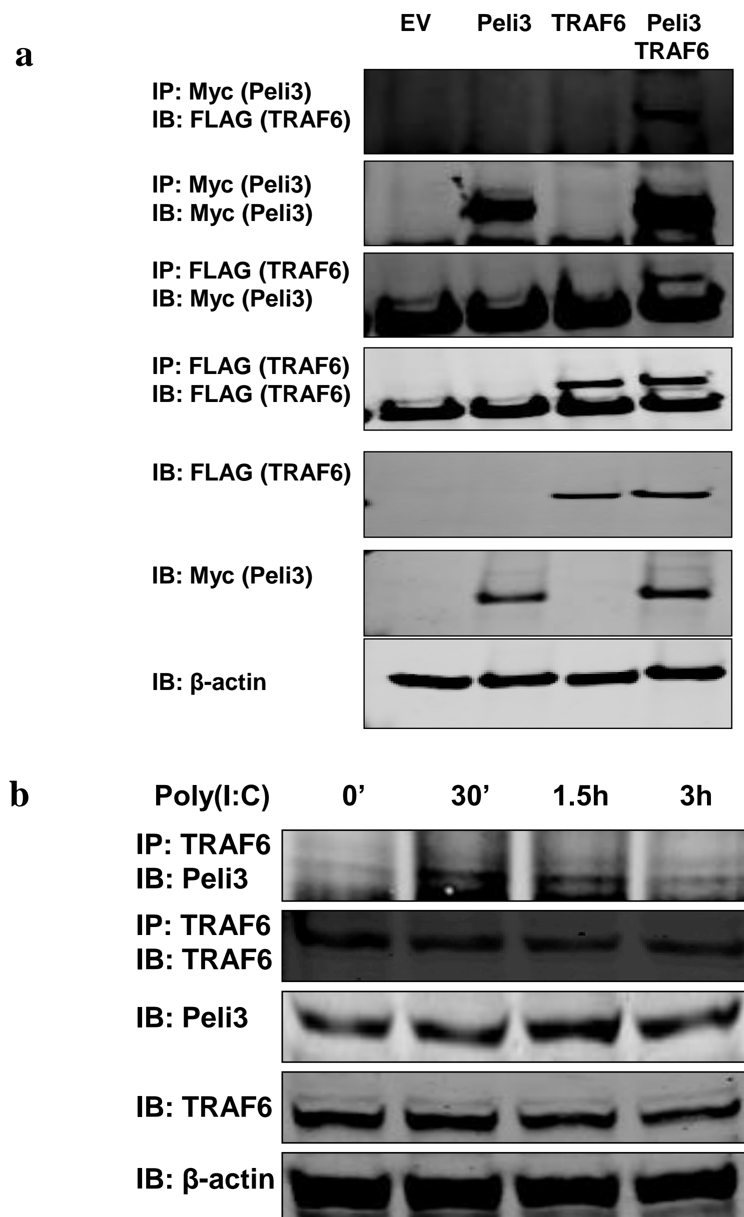

Figure 3.38 TLR3 promotes the interaction of Pellino3 with TRAF6

(a) HEKT-TLR3 cells were transfected with empty vector (EV) or expression constructs encoding Pellino3-Myc (Peli3) and/or TRAF6-FLAG. Cell lysates were generated, divided in two and immunoprecipitated with an anti-Myc or anti-FLAG antibody. Immunoprecipitated (IP) samples and whole cells lysates were subjected to immunoblot (IB) analysis for presence of Myc-tagged, FLAG-tagged proteins. β-actin was used as a loading control. **(b)** U373 cells were left untreated (0) or stimulated with Poly(I:C) (25 μg/ml) for the indicated time. Cell lysates were generated and subjected to immunoprecipitation using an anti-TRAF6 antibody. Immunoprecipitated samples and whole cells lysates were subjected to immunoblot analysis for presence of Pellino3 (Peli3), TRAF6 and β-actin was used as a loading control. Data are a representative of 3 independent experiments.

3.2.30 Pellino3 is Required for TLR3-induced TRAF6 Ubiquitination

To investigate the functional consequence of the above interaction, Pellino3 was explored as a potential E3 ligase for TRAF6 in the TLR3 pathway. MEFs isolated from WT and *Peli3*$^{-/-}$ mice were stimulated for the indicated times with Poly(I:C) and cell lysates generated. Immunoprecipitation for TRAF6 and immunoblotting analysis revealed that TLR3 did indeed induce TRAF6 polyubiquitination in WT cells (Fig. 3.39a). Interestingly however, this modification was nearly completely abrogated in the absence of Pellino3. In order to confirm this in a human model system, HEK293 cells stably expressing TLR3 were transfected with shRNA encoding either a nontargeting control shRNA or shRNA specific for Pellino3 mRNA transcript. Modest knockdown was achieved and was sufficient to lead to a complete loss of TLR3-induced TRAF6 polyubiquitination in the human cells (Fig. 3.39b). These findings suggest that the Pellino3 acts as a key mediator of TRAF6 ubiquitination during TLR3 signalling.

3.2.31 Reconstitution of TLR3-induced Polyubiquitination of TRAF6 in Pellino3-deficient Cells

Although transgenic knockout models represent the gold standard in the determination of a proteins physiological function, they are not without significant drawbacks. One such limitation was recently highlighted in which deletion by recombination of Caspase1 resulted in the deletion of the adjacent gene Caspase11 (Kayagaki *et al*., 2011). In order to investigate if the loss of TRAF6 polyubiquitination was a direct result of Pellino3-deficiency or if it was due a non-intentional genetic alteration of the mouse genome, MEFs from WT and *Peli3*$^{-/-}$ mice were isolated and infected with a MSCV encoding either a non-protein coding empty vector (EV) or a Myc-tagged murine Pellino3 expression construct. WT and *Peli3*$^{-/-}$ cells previously exposed to MSCV-EV were stimulated with Poly(I:C) for the indicated times. As observed previously, TLR3 activation caused TRAF6

polyubiquitination in a stimulus dependant manner in WT cells and TRAF6 polyubiquitination was lost in *Peli3*$^{-/-}$ cells (Fig. 3.40a). The exposure to MSCV-EV had no effect on either cell type. However, pre-exposure of cells to MSCV-Pellino3 had a profound effect on TLR3-induced TRAF6 ubiquitination. Ectopic expression of Pellino3 lead to basal ubiquitination of TRAF6 in WT cells, indicating the increase in Pellino3 protein levels can lead to TRAF6 modification. Upon TLR3 activation, an increase in this ubiquitination is evident. Importantly, in the Pellino3-deficient cells virally reconstituted with Pellino3, the stimulus induced polyubiquitination of TRAF6 is restored. Taken together, Pellino3 protein expression is a critical mediator of TRAF6 polyubiquitination in the TLR3 pathway.

3.2.32 The E3 Ligase Activity of Pellino3 is Required for TLR3-induced Ubiquitination of TRAF6

The previous findings confirm that Pellino3 is required for TLR3-induced TRAF6 polyubiquitination. To investigate if this was reliant on Pellino3's intrinsic E3 ligase activity, MEFs from *Peli3*$^{-/-}$ mice were isolated and exposed to MSCV encoding an empty vector or an expression construct encoding either Myc-tagged WT murine Pellino3 or a Myc-tagged double point mutant form of Pellino3 that lacks a functional RING-like domain. Cells were then left untreated or stimulated with Poly(I:C)) for 1.5 h and TRAF6 was immunoprecipitation and immunoblotted using an anti-ubiquitin antibody conducted. Again the WT Pellino3 reconstitution was sufficient to rescue TLR3-induced TRAF6 ubquitination (Fig. 3.41). However, the RING-like domain mutant construct of Pellino3 failed to facilitate TLR3-induced TRAF6 polyubiquitination. This data provides strong evidence suggesting that Pellino3's E3 ligase activity is essential for TLR-induced TRAF6 polyubiquitination.

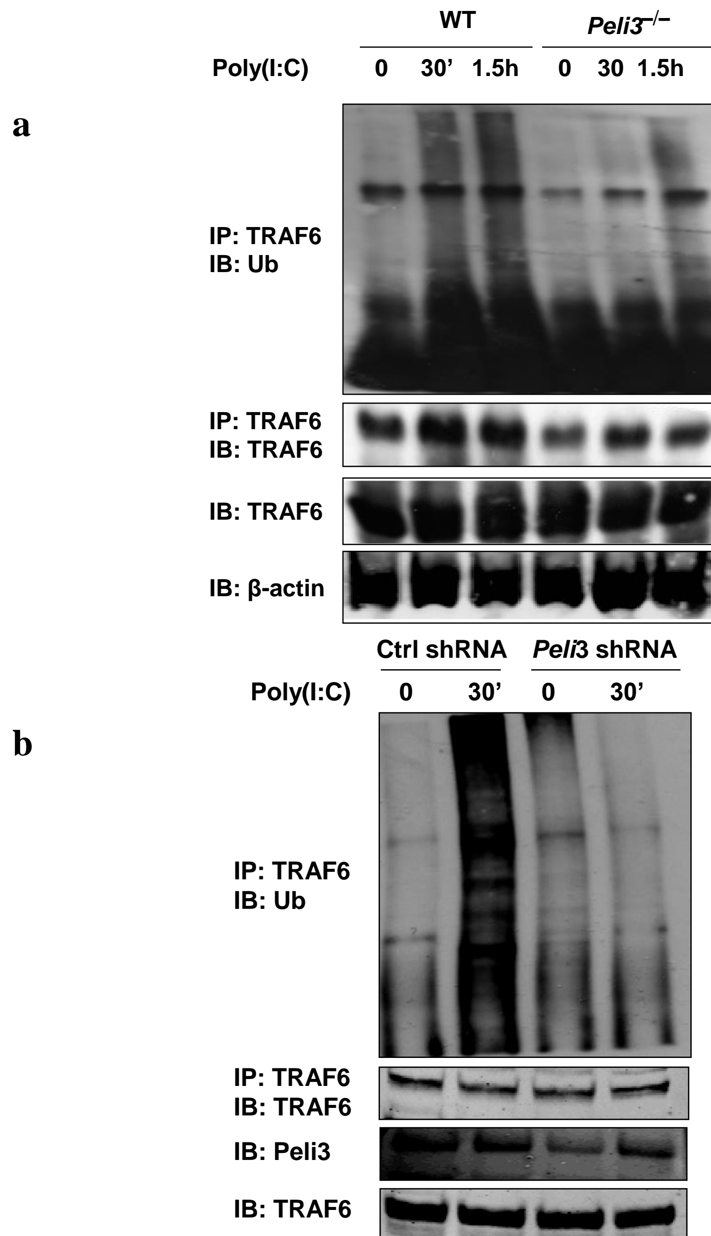

Figure 3.39 Pellino3 is required for TLR3-induced ubiquitination of TRAF6.

(a) MEFs were isolated from WT and *Peli3*$^{-/-}$ mice and left untreated (0) or stimulated with Poly(I:C) (25 μg/ml) for the indicated time. Cell lysates were generated and subjected to immunoprecipitation (IP) with an anti-TRAF6 antibody. IP samples and whole cell lysates were subjected immunoblot analysis (IB) for ubiquitin, TRAF6 and β-actin as a loading control. **(b)** HEK293-TLR3 cells were transfected with either a shRNA expression construct targeting Pellino3 (*Peli3* shRNA) or a scrambled control targeting no transcript (Ctrl shRNA) and left untreated (0) or stimulated with Poly(I:C) (25 μg/ml) for the indicated time. Cell lysates were generated and subjected to IP with an anti-TRAF6 antibody. Immunoprecipitated samples and whole cell lysates were subjected to immunoblot analysis for the presence of ubiquitin, TRAF6, Pellino3 and β-actin as a loading control. Data is representative of 3 independent experiments.

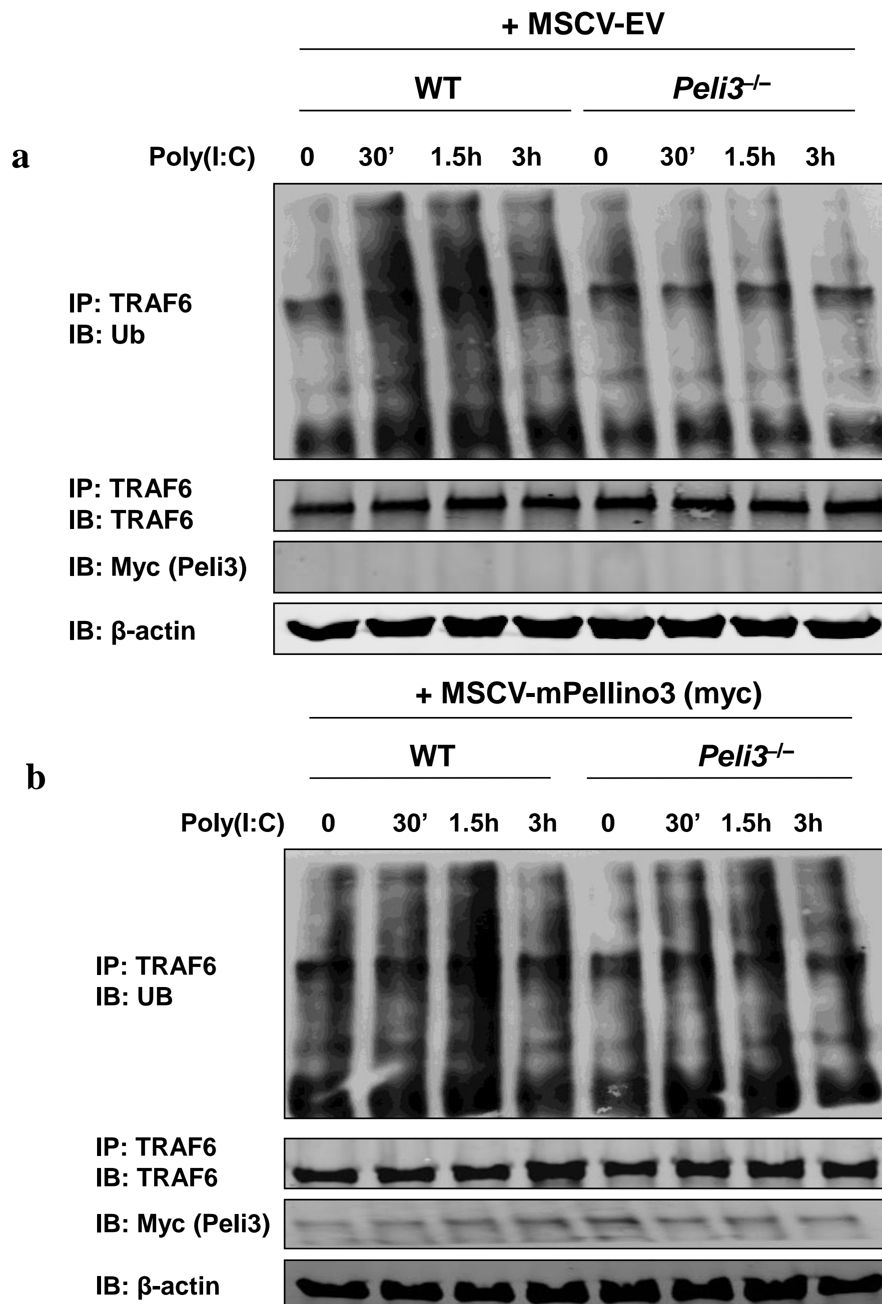

Figure 3.40 Reconstitution of TLR3-induced ubiquitination of TRAF6 ubiquitination in *Peli3*$^{-/-}$ MEFs

MEFs from WT and *Peli3*$^{-/-}$ mice were infected with Mouse Stem Cell Virus (MSCV) containing **(a)** an empty vector (EV) or **(b)** Myc-tagged murine Pellino3 (mPellino3) expression construct. Cells were then left untreated (0) or stimulated with Poly(I:C) (25 μg/ml) for the indicated times. Cell lysates were generated and subjected to immunoprecipitation (IP) with an anti-TRAF6 antibody. Immunoprecipitated samples and whole cell lysates were subjected immunoblotting (IB) for ubiquitin, TRAF6, Myc-Pellino3 (Peli3) and β-actin as a loading control. Data is representative of 3 independent experiments.

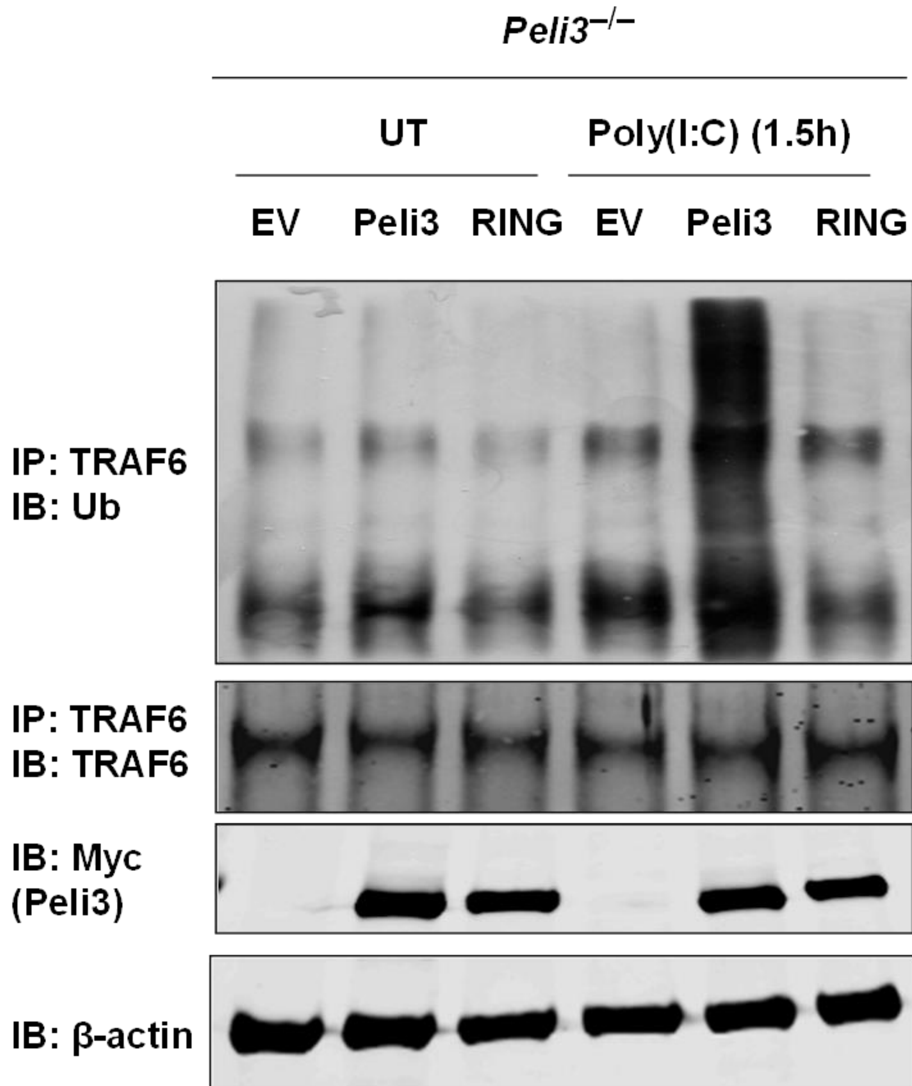

Figure 3.41 TLR3-induced ubiquitination of TRAF6 ubiquitination is dependent on the E3 ligase activity of Pellino3

MEFs from *Peli3*$^{-/-}$ mice were infected with Mouse Stem Cell Virus (MSCV) containing an empty vector (EV), myc-tagged murine WT Pellino3 (Peli3) expression construct or myc-tagged murine Pellino3 mutant construct containing a non-functional RING-like domain (RING). Cells were then left untreated (0) or stimulated with Poly(I:C) (25 μg/ml) for 1.5 h. Cell lysates were generated and subjected to immunoprecipitation (IP) with an anti-TRAF6 antibody. Immunoprecipitated samples and whole cell lysates were subjected immunoblotting (IB) for ubiquitin, TRAF6, Myc-Pellino3 (Peli3) and β-actin as a loading control. Data is representative of 3 independent experiments.

3.2.33 Pellino3 E3 Ligase Activity Inhibits TLR3-induced Type 1 IFN Production

We next exploited the above reconstitution models to assess if reconstitution of TRAF6 ubiquitination by ectopic Pellino3 in Pellino3-deficient cells was associated with negative regulatory effects on TLR3-induced expression of IFN-β. To this end, MEFs from *Peli3*$^{-/-}$ mice were left uninfected or infected with MSCV encoding an empty vector non-protein coding DNA or an expression construct for either Myc-tagged WT murine Pellino3 or a Myc-tagged mutant form of Pellino3 that lacks a functional RING-like domain. Cells were stimulated with Poly(I:C) for 6 h. To investigate if exposure of cells to MSCV-induced IFN-β mRNA expression, cells were left uninfected and infected with MSCV for 4 days prior to TLR3 stimulation and IFN-β mRNA levels examined (Fig.3.42a). Real-time PCR analysis revealed Poly(I:C)-induced IFN-β levels were comparable between cells not exposed to and pre-exposed to MSCV indicating that MSCV infection was having no effect on the experimental model of TLR3 signalling. Reintroduction of Pellino3 into the Pellino3-deficienct cells lead to a significant inhibition of Poly(I:C)-induced expression of IFN-β mRNA (Fig. 3.42a) and bioactive type 1 IFNs (Fig. 3.42b). As with TRAF6 ubiquitination, the RING-like domain mutant of Pellino3 failed to have a regulatory effect on IFN-β expression (Fig. 3.42a) or induction of bioactive type 1 IFNs (Fig. 3.42b). In order to investigate this effect in a human system, HEK293 cells stably expressing TLR3 were transfected with shRNA encoding either a nontargeting control shRNA or shRNA specific for Pellino3 mRNA transcript. Knockdown of Pellino3 lead to augmented Poly(I:C)-induced expression of IFN-β compared to control knockdown cells (Fig. 3.42). Overexpression of Pellino3 in Pellino3 knockdown cells was sufficient to regulate the Poly(I:C)-induced levels of IFN-β relative to those observed in control knockdown cells whereas overexpression of the RING-like domain mutant was ineffective in inhibiting TLR3-iduced IFN-β expression. Taken together, this data is consistent with a TLR3 signalling model in which Pellino3 mediates ubiquitination of TRAF6, blocking the interaction of TRAF6 with IRF7 and reducing ubiquitination of the latter to downregulate type 1 IFN production.

3.2.34 Polyubiquitination of TRAF6 Inhibits TRAF6-induced IRF7 Polyubiquitination

Exploration into whether ubiquitination of TRAF6 affects its capacity to ubiquitinate IRF7 was next undertaken. HEK293 cells stably expressing TLR3 were transfected with 1 μg of DNA encoding the expression constructs for either WT TRAF6, a mutant in the RING domain of $TRAF6^{(C70A)}$ which has lost its ability to conjugate polyubiquitin chains or a $TRAF6^{(K124R)}$ mutation in its primary ubiquitination site in the presence or absence of IRF7. As a control, cells were also transfected with 1 μg of non-protein coding empty vector. As both IRF7 and TRAF6 constructs were FLAG-tagged, IRF7 was immunoprecipitated selectively with and anti-IRF7 antibody and protein-protein interactions were disrupted as previously described. As expected, coexpression of WT TRAF6 with IRF7 led to an induction of polyubiquitination of the transcription factor. The point mutant in the TRAF6 RING domain resulted in a complete loss of IRF7 polyubiquitination highlighting TRAF6 as a key E3-ubiquitin ligase of IRF7. Interestingly however, mutation of the key TRAF6 ubiquitination site for its role in NF-κB-mediated signalling had no such negative affect on IRF7 ubiquitination. In fact, the lysine mutant of TRAF6 exhibits an enhanced ability to polyubiquitinate IRF7 compared to WT TRAF6. These finding highlight a novel difference in TRAF6 functionality between the different signalling pathways and suggests that polyubiquitination of TRAF6 may act as a negative regulatory signal for IRF7 activation.

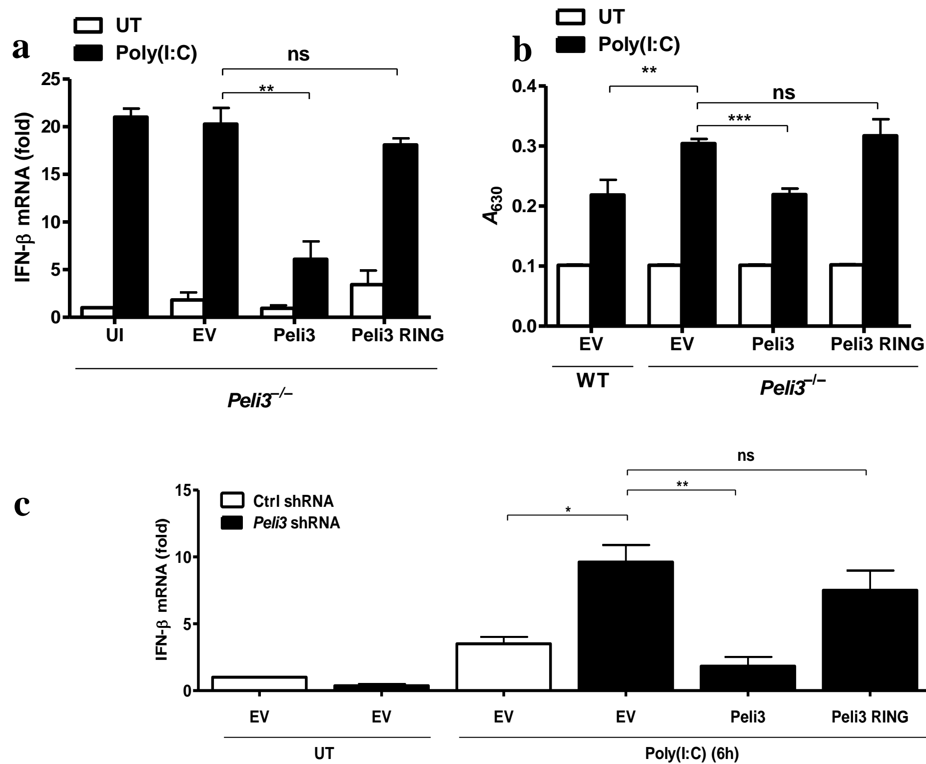

Figure 3.42 Pellino3 E3 ligase activity inhibits TLR3-induced type 1 IFN production.

(**a**) Quantitative real-time PCR of IFN-β expression in MEF cells from *Peli3*$^{-/-}$ mice were left uninfected (UI) or retrovirally infected with Mouse Stem Cell Virus (MSCV) encoding an empty vector (EV), myc-tagged murine WT Pellino3 (Peli3) expression construct or a myc-tagged murine Peli3 mutant construct containing a non-functional RING-like domain (Peli3 RING). Cells were then left untreated (UT) or stimulated with Poly(I:C) (25 μg/ml) for 6 h. (**b**) Bioactive type 1 IFN blue sensor cell assay from media from MEF cells isolated from WT and *Peli3*$^{-/-}$ mice which were retrovirally infected with Mouse Stem Cell Virus (MSCV) encoding an empty vector (EV), murine WT Pellino3 (Peli3) expression construct or murine Pellino3 mutant construct containing a non-functional RING-like domain (Peli3 RING). Cells were left untreated (UT) or stimulated with Poly(I:C) (25 μg/ml) for 24 h. (**c**) Quantitative real-time PCR of IFN-β expression in HEK293-TLR3 cells transfected with either a shRNA expression construct targeting Pellino3 (*Peli3* shRNA) or a scrambled control targeting no transcript (Ctrl shRNA). Cells were then transfected with either a non-protein coding empty vector (EV) or with WT Pellino3 (Peli3) expression construct or a Pellino3 mutant construct containing a non-functional RING-like domain (Peli3 RING). Subsequently, cells were left untreated (UT) or stimulated with Poly(I:C) (25 μg/ml) for 6 h. For real-time PCR data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. For type 1 bioassy data are presented as the absorbance at 630 nm and are the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$ **, p 0.01; ***, $p < 0.001$; ns, not significant.

3.2.35 TRAF6 Polyubiquitination during TLR3 Signalling Inhibits IFN-β Expression

In order to investigate if Pellino3 meditated TRAF6 polyubiquitination is the mechanism by which Pellino3 limits TLR3-induced type 1 interferon production, HEK293 cells stably expressing TLR3 were subjected to TRAF6-specific knockdown and then reconstituted with ectopic WT TRAF6 or a point mutant of TRAF6 (K124R) that is refractory to ubiquitination. Cells were stimulated with Poly(I:C) for 6 h and the mRNA extracted. Knockdown of TRAF6 led to a near complete loss in Poly(I:C)-induced IFN-β highlighting TRAF6's key role in the type 1 IFN pathway (Fig. 3.44a). WT TRAF6 partially rescued TLR3-induced IFN-β but the RING mutant of TRAF6 showed greater efficacy in this endeavour suggesting that the ubiquitination of TRAF6 is a negative signal in the context of activation of TLR3-induced expression of type 1 IFNs. In order to investigate this mutant in pro-inflammatory signalling, TLR3-induced IL-6 was also examined (Fig. 3.44b). Again, TRAF6 knockdown led to a significant decrease in IL-6 expression and this was completely rescued with reconstitution of WT TRAF6. In line with previous studies, the TRAF6 mutant failed to rescue IL-6 protein levels indicating TRAF6 polyubiquitination is a positive signal with respect to TLR3-induced expression of pro-inflammatory cytokines.

These data further our mechanistic understanding of not only Pellino3's physiological role in TLR signalling but also highlights a novel pathway in TLR3-induced type 1 IFN production. TLR3 signalling leads to an upregulation of Pellino3 and its interaction with TRAF6. Pellino3's E3 ligase actively facilitates TLR3-induced polyubiquitination of TRAF6. This modification acts as a regulatory signal inhibiting TRAF6-IRF7 interaction and IRF7 polyubqitination. Loss of IRF7 polyubiquitination results in downregulation of type 1 IFN gene expression.

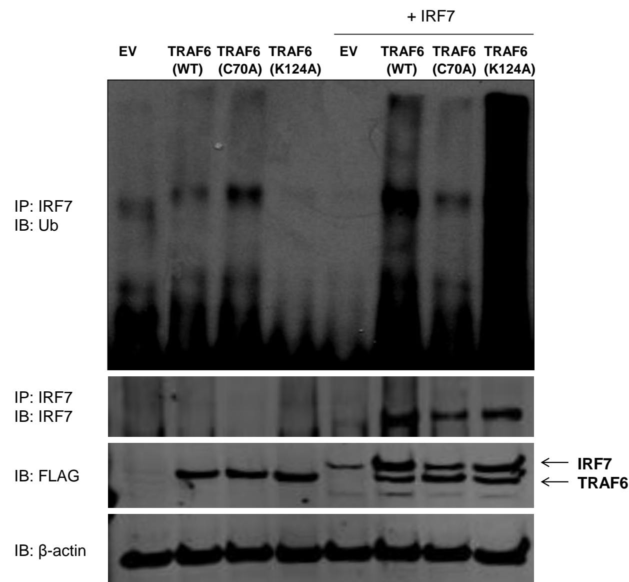

Figure 3.43 TRAF6 ubiquitinates IRF7.

HEKT-TLR3 cells were transfected with empty vector (EV), or expression constructs encoding a FLAG-tagged TRAF6 (WT), a FLAG-tagged TRAF6 mutant construct containing a non-functional RING-like domain ($TRAF6^{(C20A)}$), a FLAG-tagged TRAF6 mutant construct that is refractory to ubiquitination ($TRAF6^{(K124)}$) and/or a FLAG-tagged IRF7 expression construct. Cell lysates were generated and subjected to immunoprecipitation with an anti-IRF7 antibody. Immunoprecipitated (IP) samples and whole cell lysates were subjected immunoblotting (IB) for ubiquitin, IRF7, FLAG and β-actin as a loading control. Data is representative of 3 independent experiments.

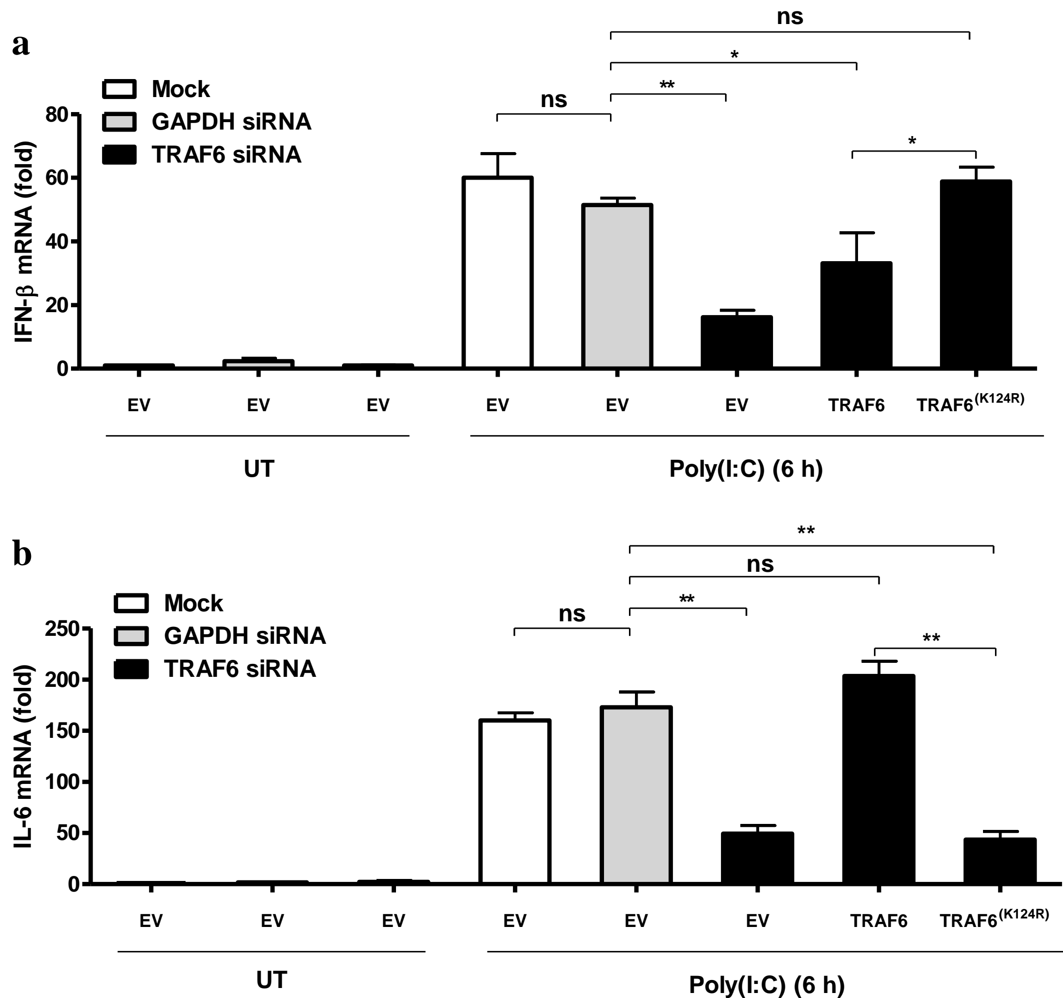

Figure 3.44 Differential effects of ubiquitination of TRAF6 on TLR3-induced expression of IFN-β and IL-6.

HEK293-TLR3 cells were left untransfected (Mock) or transfected with siRNA targeting GAPDH or TRAF6. Cells were subsequently transfected with a non-protein coding empty vector (EV) or an expression constructs encoding WT TRAF6 (TRAF6) or an expression construct encoding a TRAF6 mutant construct that is refractory to ubiquitination ($TRAF6^{(K124R)}$). Cells were left untreated (UT) or stimulated with Poly(I:C) (25 μg/ml) for 6 h and subjected to quantitative real-time PCR analysis for **(a)** IFN-β and **(b)** IL-6. Data are presented as a fold relative to WT UT, normalised to HPRT expression and as the mean +/- S.E.M. of three independent experiments and were subjected to paired two-tailed Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

Chapter 4:

Discussion

4.1 Discussion

The rapid initiation of type 1 IFNs by innate immune cells represents a key defence strategy to mediate viral eradication from the host. Recognition of the nucleic acids present in the viral genome or generated during viral replication has evolved as the major mechanism that innate immunity employs to trigger the type 1 IFN protective response. Central to this detection and response are the PRRs. TLRs ligate viral motifs and instigate downstream signalling cascades to activate specific transcription factors to induce expression of these anti-viral mediators. However, type 1 IFNs, if left unchecked, can mediate a number of deleterious outcomes to the host. It is now well established that elevated serum levels of IFNs are found in patients with autoimmune diseases such as SLE and rheumatoid arthritis, with increased levels correlating with disease activity (Hooks *et al*., 1979). Furthermore, IFN-α/β therapies can also directly induce autoimmune phenotypes which include SLE-like disorders (Ronnblom *et al*., 1991). Recently, a mechanistic understanding of how aberrant type 1 IFNs mediate these pathologies has begun to be realised. Elevated type 1 IFNs can break peripheral tolerance by the activation of immature DCs which in turn can present self-antigen from dying host cells initiating the auto-reactive B and T cells that characterise disorders like SLE (Blanco *et al*., 2001). A strict balance between the beneficial viral clearance properties of type 1 IFNs must be weighed against the acute and chronic pathologic conditions associated with this dysregulated production. Therefore, tight regulation of TLR-induced type 1 IFNs is crucial to prevent detrimental levels of cytokine accumulation after viral clearance. For this reason, investigation and mechanistic understanding of the regulatory signalling circuitry activated by TLRs is a vital prerequisite to potential medical intervention to both viral infection and host autoimmunity.

The aim of this study was to characterise the physiological role of Pellino3 in TLR signalling. In order to achieve this goal, the generation of a gene targeted disruption of the murine *Peli3* gene was commissioned. Deletion of the third protein coding exon of *Peli3* results in a frameshift in its remaining downstream exons resulting in a Pellino3-deficient animal. Peli3$^{-/-}$ mice were viable, developmentally normal and

displayed no obvious immuno-deficiencies when housed in specific pathogen free housing. Furthermore, to date, there is no evidence of the spontaneous induction of autoimmune disease as exhibited in *Peli1*$^{-/-}$ mice (Chang *et al.*, 2011), found to occur in Pellino3-deficient animals. This important observation highlights non-redundant roles for the highly conserved mammalian Pellino proteins in control of the adaptive immune response. The data provided in this thesis indicates that Pellino3 has no role in TLR induction of pro-inflammatory cytokines but plays a key regulatory role in controlling TLR3-dependent induction of type 1 IFNs in response to viral dsRNA and live viral infection. Pellino3 upregulation during TLR3 signalling facilitates its interaction with TRAF6, an essential signal for TRAF6 polyubiquitination. In this novel auto-regulatory signalling network, polyubiquitinated TRAF6 can no longer bind to or catalyse IRF7 modification thereby terminating the type 1 IFN response.

Prior to this work, the role of Pellino3 in the innate immune responses was investigated using overexpression and RNAi studies. Such approaches implicated Pellino3 in contrasting roles in both positive and negative regulatory functions in inflammatory signal activation of NF-κB and the MAPKs (Xiao *et al.*, 2008, Butler *et al.*, 2005). However, studies into the Pellino proteins in a physiologically setting had been restricted to Pellino1. Pellino1 was originally shown to play a critical role in TRIF-dependent activation of NF-κB and subsequent pro-inflammatory cytokine production during TLR activation (Chang *et al.*, 2009). Interestingly, Pellino3 also functions in the TRIF-dependent signalling cascade. However, Pellino3 has no effect on pro-inflammatory cytokine expression and instead mediates regulation of TRIF-dependent signal activation of the type 1 IFNs, the other arm of the TLR3 response. Pellino3 was found to specifically function in the TLR3 sub-pathway that induces of type 1 IFNs and genes such as IP-10 that are similarly regulated at the transcriptional level. The observation that Pellino3 negatively regulates TRIF-mediated induction of IFN-β is in agreement with a recent study involving oxLDL derived from atherosclerotic lesions (Tzieply *et al.*, 2012). In this study oxLDL signals via the scavenger receptor-A1 (SR-A1) and leads to IRAK-induced activation of Pellino3 and ubiquitination of TANK resulting in inhibition of TBK1-mediated activation of IRF3. Such findings in conjunction with the present study highlight a role for Pellino3 in a regulatory system that controls production of type 1 IFNs. As type 1

IFNs have been implicated in promoting atherosclerosis (Goossens *et al*., 2010), experimental investigation into models of this disorder in the Pellino3-deficient mice may provide genetic evidence for a role for Pellino3 in non-viral, and TLR3-dependent regulation of type 1 IFNs through distinct intracellular mechanisms. This is a very interesting concept in light of the fact that Pellino1 facilitates IRF3 activation of IFN-β in response to RLR receptor RIG-I activation (Enesa *et al*., 2012). One could speculate that the Pellino molecules may act in synchronism to control the magnitude and temporal kinetics of a type 1 IFN response. Therefore, investigation into the interplay between these two non-redundant family members may further our understanding of anti-viral signalling by dsRNA recognition. Although the upstream kinases of Pellino1 have been identified as TBK1 and IKKε (Goh *et al*., 2012), and TBK1 is required for Pellino3 upregulation, the role of upstream kinases for Pellino3 phosphorylation is still unknown in TLR3 signalling. This deficit in our understanding of TLR3 signalling requires further attention as phosphorylation is known to enhance Pellino protein E3 ligase activity (Ordureau *et al*., 2008). One potential kinase that may serve this purpose is IRAK1, as it has been shown to phosphorylate Pellino proteins *in vitro* (Ordureau *et al*., 2008). In addition, a recent report has indicated that IRAK1 can inhibit TLR3 activation of type 1 IFNs and although IRAK1 has other targets and can also inhibit pro-inflammatory cytokine production, Pellino3 phosphorylation may be one of the mechanisms it employs to inhibit TLR3 signalling (Bruni *et al*., 2013). Other potential avenues of investigation into how Pellino1 and Pellino3 mediate antagonistic control of type 1 IFN may be explored with inspection of the primary structure of Pellino3. Pellino3 contains an additional 27 aa leader sequence at its N terminus not on either Pellino1 or Pellino2, and otherwise Pellino3 exhibits high sequence homology with the other family members (~82% aa identity without the leader sequence). The role for this additional sequence is still unknown and it may facilitate Pellino3-specific interactions with substrates, kinases, E2 ubiquitin conjugating enzymes or influence its expression, protein stability or cellular localisation. The delineation of the function of this leader sequence will represent a further appreciation of the role of Pellino3 in immunobiology.

Recently, Pellino1 has also been implicated in the activation of the MAPK pathways in the CNS by TLR2 and TLR4 signalling (Xiao *et al.*, 2013). RNAi and overexpression studies have also implicated a requirement for Pellino3 in TLR2 and TLR4-induced activation of p38 MAPK signalling (Mellett *et al.*, 2011, Butler *et al.*, 2005). However, experimental evidence provided here indicates that Pellino3 has no function in pro-inflammatory cytokine production in response to the TLR2 and TLR4 agonists; Pam_2CSK, Pam_3CSK, Zymosan and LPS. In addition, Pellino3-deficiency has no adverse effect on TLR3-induced activation of MAPK pathways questioning its role, if any, of Pellino3 in MAPK signalling. These discrepancies may again in part be explained by the high sequence homology between the two family members. Overexpression of Pellino3 may force its interaction with upstream proteins and mediate ubiquitination of Pellino1's substrates; however in a endogenous physiological environment, Pellino3 may not be recruited to the given signalling intermediate. Additionally, RNAi although very specific may also lead to knockdown of very similar transcripts, like that which are present between Pellino1 and Pellino3 and careful selection of the interfering sequence is vital for specific knockdown (Schwarz *et al.*, 2006, Ui-Tei *et al.*, 2004). Therefore, it is possible that some of the roles attributed to Pellino3 by overexpression and RNAi approaches may in fact be facilitated by Pellino1 and may explain why Pellino3-deficiency *in vivo* has no effect on NF-κB and MAPK activation during TLR signalling. In addition, during RNAi knockdown of Pellino proteins, some functional compensation may be exhibited by the other family members obscuring the individual proteins functions.

In order to define if the augmentation of type 1 IFNs exhibited in the *Peli3*$^{-/-}$ mice during anti-viral signalling, played a physiologically relevant role *in vivo*, mice were infected with EMCV, a small non-enveloped single stranded RNA virus that produces dsRNA during replication and is recognised by TLR3 (Carocci and Bakkali-Kassimi, 2012). EMCV infection primarily targets the heart and brain of the host and TLR3-induced expression of type 1 IFNs is known to protect against EMCV as TLR3-deficient animals are more susceptibility to EMCV-induced lethality and have higher viral titres and increased pathological damage in their cardiac tissue upon EMCV infection (Hardarson *et al.*, 2007a). Conversely, *Peli3*$^{-/-}$

mice displayed increased survival and EMCV clearance from the heart. In addition, *Peli3*$^{-/-}$ mice did not exhibit the pathological alterations seen in the hearts of control animals and this collated to decreased inflammation but increased IFN-β expression in the knockout animals. Such findings are consistent with Pellino3 playing a critical role in the control of TLR3-induced type 1 IFN production and this increased production facilitating enhanced viral elimination from the murine host. Interestingly, the major organs of EMCV infection are the heart and brain, two tissues with the highest expression of Pellino3 mRNA (Jensen and Whitehead, 2003b). It could be speculated that viruses such as EMCV have shaped Pellino3 evolutionary conservation in the mammalian genome, by controlling aberrant and even harmful levels of type 1 IFN during and after viral infection by orchestrating host homeostasis and resolution of the anti-viral immune response.

Although TLR3 signalling is critical for resistance against EMCV, a member of the RLR pathway MDA-5, can also recognise EMCV and Poly(I:C) dsRNA *in vivo* and plays a significant role in type 1 IFN production and anti-viral protection (Gitlin *et al.*, 2006, Kato *et al.*, 2006). Therefore, it cannot be ruled out that the increased type 1 IFN exhibited in the Pellino3-deficient animals may in part be facilitated by Pellino3 negatively regulating the MDA-5 pathway. However, in our experimental models, both Poly(I:C) and EMCV-induced the expression of Pellino3 mRNA in a TRIF-dependent manner. Interestingly, both activators could still induce upregulation of Pellino3 mRNA in the absence of the essential MDA-5 adaptor MAVS, indicating that TLR3- and TRIF-dependent signalling was responsible for Pellino3 upregulation and negative feedback inhibition. As direct application of Poly(I:C) is used throughout this study, it was important to distinguish which PRR was being triggered by this mode of stimulation. Although both MDA-5 and TLR3 can also recognise Poly(I:C), MDA-5 requires direct delivery of the dsRNA into the cytosol where the receptor is present. Importantly, although transfected Poly(I:C) induces IFN-β production in WT cells, no further augmentation is induced in *Peli3*$^{-/-}$ cells. This suggests that Pellino3 does not regulate MDA-5 signalling. In addition, direct treatment of Poly(I:C) activates cell surface TLR3 and in the case of intracellular TLR3, the dsRNA is internalised by the Clathrin-mediated endocytic pathway for endosomal delivery whereby it bypasses the cytosolic MDA-5 (Itoh *et*

al., 2008). Furthermore, direct application of Poly(I:C) upregulates type 1 IFN production in a TRIF-dependent manner. Deficiency of MAVS has no effect on exogenously administrated Poly(I:C)-induced IFN-β. These data provide strong evidence that TLR3-mediated type 1 IFN production is targeted by Pellino3. It was also considered that the increased survival of Pellino3-deficient mice *in vivo* may be due to a secondary role of Pellino3 in inhibiting type 1 IFN signalling. This however does not seem to be the case as IFN-α4-induced phosphorylation of STAT1 and STAT3 displayed similar activation kinetics in BMDCs stimulated *ex vivo* from WT and Pellino3-deficent mice. Although this does not completely rule out an effect of Pellino3 on IFNAR signalling during the complexities of an *in vivo* viral infection, it does suggest a non-essential role for Pellino3. Taken together, this data reaffirms that Pellino3 is targeting the TLR3 proximal pathway specifically and mediates a physiological relevant role during lethal viral infection.

The data presented in this thesis illustrates a novel negative regulatory system whereby Pellino3 is upregulated during TLR3 signalling in order to terminate type 1 IFN production. In this model, Pellino3 functions by negatively regulating IRF7 ubiquitination, nuclear translocation and activation. Interestingly, although IRF7 is the key mediator of type 1 IFNs in pDCs, Pellino3 deficiency had no effect on TLR –signalling in this specialised anti-viral cell type. However, as pDC do not express TLR3 or signal through TRIF and instead only utilise Myd88 dependent signalling (Honda *et al*., 2005a), it is plausible that Pellino3 is not upregulated in this cell type during TLR activation and therefore cannot mediate negative regulation of the pathway. Interestingly, during TLR3 signalling the absence of Pellino3 had no effect on TBK1 activation or IRF3 phosphorylation. However, levels of IRF7 polyubiquitination were greatly increased in the absence of Pellino3 giving rise to two hypotheses. The first was that Pellino3 was acting as a direct DUB enzyme for IRF7 with Pellino3-mediated deubiquitination of IRF7 serving to inhibit its transcriptional activity. However there is no evidence to suggest that Pellino3 exhibits DUB activity. The other potential mechanism by which Pellino3 negatively regulated IRF7 polyubiquitination was by interfering with the E3 ligase that ubiquitinates IRF7. IRF7 is reported to be the master regulator of type 1 IFN in TLR7/9 signalling in pDCs (Honda *et al*., 2005a), with TRAF6 E3 ligase activity

being essential for the IRF7 antiviral response (Kawai *et al.*, 2004). Although TRAF6s role in TLR3-induced NF-κB and MAPK activation is still a matter of contention (Gohda *et al.*, 2004, Sato *et al.*, 2003), it stood to reason that TRAF6 may serve a specific function for IRF7 similar to that displayed during intracellular TLR7/9 signalling. Furthermore, TRAF6 polyubiquitination and activation of IRF7 was also reported to be induced by the Epstein-Barr virus LMP1. During LMP1 activation, polyubiquitination of the last 3 lysine residues of IRF7 by TRAF6 was shown to be a prerequisite for IKKε mediated phosphorylation and activation of IRF7 transcriptional activity (Ning *et al.*, 2008b). The present investigations into TRAF6 and IRF7 during TLR3 signalling reveal that a stimulus dependent interaction between the E3 ligase and the type 1 interferon transcription factor indeed occurred upon dsRNA recognition. Importantly, Pellino3 serves as a potent negative regulator of this interaction as increased association is evident in Pellino3-deficient cells. Taken together, such findings explain not only the increased ubquitination and activity of IRF7 but also the increased serine phosphorylation observed in TLR3 activated Pellino3-deficient cells. Interestingly, the naturally occurring Thogoto virus encodes a virulence factor known as the ML protein that also inhibits the TRAF6-IRF7 interaction to circumvent host immunity (Buettner *et al.*, 2010). This highlights the critical nature of TRAF6 mediated IRF7 activation for type 1 IFN production. It is therefore not surprising that a physiological regulatory mechanism has co evolved to target this intersection to limit TLR3 mediated signalling.

In order for Pellino3 to negatively regulate type 1 IFN by modulating TRAF6 activation of IRF7, it would therefore have to be assumed that TRAF6 is required for TLR3 activation of type 1 IFNs. As TRAF6s role in TLR3 signalling is still somewhat unclear, it was important to confirm the published observations that TRAF6 is essential for Poly(I:C) and EMCV-induced type 1 IFN production (Konno *et al.*, 2009). TRAF6-deficient cells displayed a complete loss of TLR3 induction of IFN-β expression in line with this previous report. In addition, ablated TLR3-induced IFN-β expression could be rescued by reconstitution of WT TRAF6 but not the C70A E3 ligase dead TRAF6 mutant. These data indicate that TRAF6 and its E3 ligase activity is vital for TLR3 induction of IFN-β. In confirmation with the seminal description of Pellino3, Pellino3 and TRAF6 interaction was observed in an artificial

overexpression system (Jensen and Whitehead, 2003c). A critical finding in this study demonstrated that under physiological relevant signalling conditions, TLR3 activation results in Pellino3 association with TRAF6 in a stimulus dependent manner. Importantly, this interaction is crucial for TRAF6 polyubiquitination and absence of Pellino3 results in a complete abrogation of TLR3-induced TRAF6 modification. As TRAF6 polyubiquitination can be rescued by retroviral reconstitution with WT Pellino3 but not an E3 ligase activity dead mutant, it provides strong evidence that Pellino3 acts as an E3 ubiquitin ligase for TRAF6 during TLR3 signalling. Furthermore, Pellino3s E3 ligase activity is the critical mediator of its negative regulatory effects on type 1 IFN production. This indicates that Pellino3 polyubiquitination of TRAF6 serves to inhibit TRAF6 activation of IRF7. Therefore it is possible that Pellino3 may act as the direct E3 ligase for TLR3-dependent TRAF6 polyubiquitination or Pellino3 may polyubiquitinate another E3 ligase critical for TRAF6 polyubiquitination. However, this does not preclude the possibility that Pellino3 may function to facilitate TRAF6 auto-ubiquitination. Interestingly, TRAF-interacting protein with a forkhead-associated domain (TIFA) has been shown to play a vital role in TRAF6s ability to autophosphorylate and undergo oligomerisation, properties classically associated with TRAF6's intrinsic ability to activate the TAK1-IKK complex in NF-κB signalling (Lamothe *et al.*, 2007a, Baud *et al.*, 1999). TIFA interaction with TRAF6 is dependent on the formers FHA domain, and results in the recruitment of the E2 ligases Ubc13–Uev1A that promote TRAF6 catalytic activity (Ea *et al.*, 2004). As Pellino3 also contains a FHA domain, it may associate with TRAF6 and function in similar manner to TIFA during TLR3 stimulation. Alternatively, it is possible that TLR3 stimulation results in the formation of a Pellino3, TRAF6 and TIFA complex whereby Pellino3 mediates polyubiquitination of TIFA or an associated protein to facilitate TIFA dependent TRAF6 auto-ubiquitination. It is interesting that Pellino3-induced polyubiquitination of TRAF6 acts as a negative regulatory signal without inducing TRAF6 proteasomal degradation. Recently Pellino1 has been shown to bind TRAF3 and mediate its K48-linked polyubiquitination to allow TLR-induced MAPK activation (Xiao *et al.*, 2013). It is intriguing that both Pellino proteins are involved in TRIF-dependent signalling and TRAF protein modification in different TLR pathways and the elucidation of the signalling events or cell type restrictions that

mediate these phenomena will be fascinating. Importantly, a number of critical functions of the Peli proteins may be hidden due to functional redundancy within the proteins and the generation of double and triple knockout animals may reveal why 3 highly related proteins have been so highly conserved throughout mammalian evolution.

Although the exact molecular interactions require further interrogation, what is certain is that Pellino3 E3 ligase activity facilitates TRAF6 polyubiquitination and this acts a negative regulatory mechanism preventing TRAF6 activation of IRF7. As polyubiquitination of TRAF6 is classically associated with its ability to activate downstream signalling cascades by the formation of a signalling complex involving TAK1 and IKK, the above findings are highly original. Emerging data in the field is indicating that TRAF6 can also activate NF-κB in some cases in the absence of auto-ubquitination and it can also catalyse the formation of free polyubiquitin chains to serve this signalling platform purpose (Walsh *et al*., 2008, Xia *et al*., 2009). It is likely that polyubiquitinated TRAF6 cannot bind IRF7 to high affinity as loss of TRAF6 polyubiquitination is associated with increased IRF7 interaction. Alternatively, oligomerisation of TRAF6 may mask the IRF7 binding site preventing its association and activation of the transcription factor. By employing a cell-based overexpression ubiquitination assay, it is clear that TRAF6-induced polyubiquitination of IRF7 is critically dependent on TRAF6 E3 ligase activity but not on TRAF6's own posttranslational modification. In fact, TRAF6s own polyubqquitination site (K124) serves as a negative regulatory site for TLR3-induced IFN-β, while this same site is essential for TLR3-mediated activation of the NF-κB responsive classic pro-inflammatory cytokine IL-6. These findings also give rise to the model that the auto-ubiquitination of TRAF6 may serve as a signalling divergence point for TLR activation of pro-inflammatory and anti-viral cytokine production.

In conclusion, the generation of Pellino3-deficient mice has elucidated a specific and non-redundant physiological role for Pellino3 in TLR biology. Upon TLR3 recognition of viral dsRNA, type 1 IFNs are produced to combat viral infection. Although vital for host protection and viral clearance, type 1 IFNs must be tightly

controlled as excessive production can directly lead to chronic and acute autoimmune disease. This thesis describes the identification of a novel auto-regulatory mechanism employed by TLR3 during anti-viral signalling. TLR3 upregulates Pellino3 to inhibit type 1 IFN transcription. Pellino3 specifically counter regulates TLR3 activation of IRF7. In order to control this process, Pellino3 facilitates polyubiquitination of TRAF6, a modification that prevents TRAF6 association with and activation of IRF7 (summarised in Fig. 4.1). These findings suggest that Pellino3 may be a critically important and protective protein utilised to defend the body against type 1 IFN-mediated autoimmunity. In order to build upon these findings, investigation into Pellino3s functionality in patients with autoimmune disorders in conjugation with experimental disease models in the knockout mice is required. Such exploration could provide important and potential clinically relevant insight into the role of the Pellino3 in the physiological regulation of viral and autoimmune disease.

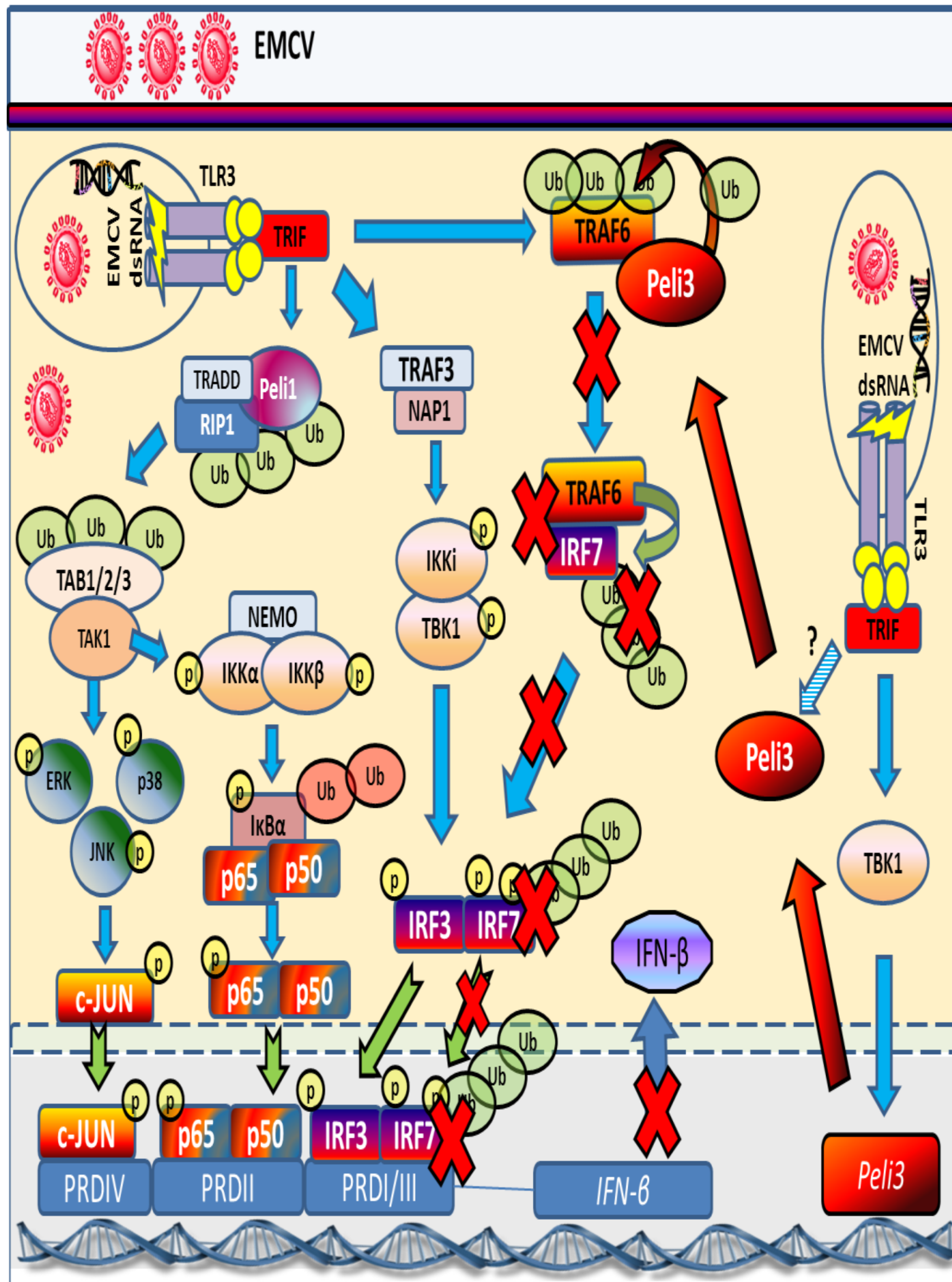

Fig. 4.1 Pellino3 regulates TLR3-induced type 1 IFN

Yellow circles marked with a “p” inside represent phosphorylation events, green circles with “Ub” represents K63-linked ubiquitination while red circles with “Ub” represent K48-linked ubiquitination. Straight Blue arrows represent TRIF-dependent signalling. Straight red arrows indicate Pellino3 signalling. Straight green arrows represent nuclear translocation. The stripped blue arrow indicates possible TRIF signalling. A red “X” indicates Pellino3 inhibition of TLR3-induced IFNβ.

Chapter 5:

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