Investigating new roles of the ubiquitin proteasome system in cell death and innate immune signalling

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CONTENTS

ABSTRACT

Immune responses are a complex network of interactions between proteins in different cellular processes with multiple layers of regulation. In the present study, we explored the role of some regulators of the ubiquitination and proteasome systems in cell death and innate immune signalling pathways in macrophages. Initial studies focused on a form of regulated cell death termed necroptosis, a type of cell death mediated by the receptor-interacting protein kinases (RIP). RIP kinases are known to interact with E3 ubiquitin ligases Pellino proteins. Pellino 1 has recently been described to target RIP kinases and regulate necroptosis. Firstly, the roles of the E3 ubiquitin ligases Pellino 2 and Pellino 3 were studied. The findings demonstrated that neither of them regulates necroptosis induced by innate immunity triggers. Studies next characterised the role of TLR signalling pathways in the activation of hypoxia-inducible factor 1α (HIF-1α), a critical target of the ubiquitin/proteasome system. Interestingly, these studies revealed that some of the TLRs promote a faster migration form of HIF-1α of approximately 40kDa. The upregulation of the latter was mediated by the TLR adaptor protein TRIF. The role of proteasome and lysosome mediators on processing of full length 110kDa HIF-1α to this smaller form was also assessed. As part of these experimental approaches, an intriguing discovery was made showing that treatment of macrophages with the proteasome inhibitor MG132 induced high levels of the anti-inflammatory cytokine IL10. This effect was dependent on P38 MAPK pathway activation and phosphorylation of the transcription factor CREB. Similar results were seen with proteasome inhibitor Bortezomib which is used clinically in the treatment of multiple myeloma. This effect is interesting considering that IL10 is a growth factor for myeloma cells and some patients treated with Bortezomib develop a refractory response to treatment and progression of disease. Overall, the present research provides novel insights into the roles of some regulators of the ubiquitin and proteasome systems in cell death and inflammatory signalling in macrophages.

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"To put the world right in order, we must first put the nation in order; to put the nation in order, we must first put the family in order; to put the family in order, we must first cultivate our personal life; we must first set our hearts right."

Confucius

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"*I am grateful to have been loved and to be loved now and to be able to love, because that liberates. Love liberates. It doesn't just hold—that's ego. Love liberates. It doesn't bind. Love says, 'I love you. I love you if you're in China. I love you if you're across town... I love you.'"*

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ABBREVIATIONS

2-DOG	2-Deoxy-D-glucose
ANG2	Angiopoietin-2
AP1	Activator protein 1
APAF1	Apoptotic protease-activating factor 1
APS	Ammonium persulfate
Arnt	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
BCL-2	Anti-apoptotic members of the B cell lymphoma 2
bHLH	Basic helix–loop–helix/Per-Arnt-Sim homology
BMDCs	Bone marrow derived dendritic cells
BMDMs	Bone-Marrow derived Macrophages
BME	β-mercaptoethanol
BSA	Bovine serum albumin
CAD	C-terminal transactivation domain
CBP	CREB-binding protein
CCL27	C-C motif ligand 27
CCR10	C-C chemokine receptor type 10
CHX	Cycloheximide
cIAP1/2	Cellular inhibitor of apoptosis protein 1 and 2
COX-2	Cyclooxygenase 2
CREB	cAMP response element-binding protein
CT	Cycle Threshold
CTCL	Cutaneous T cell lymphoma
CXCL1	C-X-C motif ligand 1
CXCL10	C-X-C motif chemokine 10
CXCL2	C-X-C motif ligand 2
CYLD	Deubiquitinases cylindromatosis
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Damage-associated molecular patterns
DCs	Dendritic cells
DD	Death domain
DISC	Death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMOG	Dimethyloxaloylglycine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
DTT	1,4-Dithiothreitol
DUSP1	Dual specificity protein phosphatase 1
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum

ERK	Extracellular signal-regulated kinases
FADD	FAS associated death domain protein
FASL	Fas ligand
FBS	Fetal Bovine Serum
FHA	Forkhead-associated domain
FIH-1	Factor-inhibiting HIF-1α
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GLUT-1	Glucose transporter 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSK	GlaxoSmithKline
GSK3	Glycogen synthase kinase 3
HBS	HIF-binding site
HIF	Hypoxia-inducible transcription factors
HOIL-1	Haem-oxidized IRP2 ubiquitin ligase-1
HOIP	HOIL-1 interacting protein
HPRA	The Health Products Regulatory Authority
HSP70	Heat-shock protein 70
HSP90	Heat-shock protein 90
IAPs	Inhibitors of apoptosis proteins
IBD	Inflammatory bowel disease
iBMDMs	immortalized BMDMs
IFNs	Interferons
IFNγ	Interferon γ
IKK	IκB kinase
IKK1/2	IκB kinases 1 and 2
IL10	Interleukin (IL)-10
IL10R	Interleukin (IL)-10 receptor
IL-1beta	Interleukin 1 beta
IL6	Interleukin (IL)-6
IPAS	Inhibitory PAS domain protein
IRAK	Interleukin-receptor-associated kinase
IRFs	Interferon Regulatory Factors
JAK	Janus kinase
JNK	N-terminal kinase
LDH	Lactate dehydrogenase
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin chain assembly complex
MAL	MYD88-adaptor-like protein (also TIRAP)
MAPK	Mitogen-activated protein kinases
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
M-CSF	Macrophage-colony stimulating factor
Mda-5	Melanoma differentiation associated factor 5
MECL-1	Multicatalytic endopeptidase complex-like 1

MEF	Mouse embryonic fibroblasts
MG	Myasthenia gravis
MHC class I	Major histocompatibility comlex class I
MLKL	Mixed lineage kinase domain-like protein
MM	Mutiple Myeloma
MMP 9	Matrix metallo proteinase 9
MyD88	Myeloid differentiation primary-response protein 88
Na3VO4	Sodium orthovanadate
NACHT	Nucleotide-binding oligomerization
NAD	N-terminal transactivation domain
NaF	Sodium fluoride
NCCD	Nomenclature Committee on Cell Death
NEMO	Nuclear Factor Kappa B Essential Modifier
NK	Natural killer
NLRs	Nucleotide-binding oligomerization-like receptors
NLS	Nuclear localization signal
NOD2	Nucleotide-binding oligomerization domain containing 2
NOS2	Nitric oxide synthase
OD	Optical Depth
ODDD	Central oxygen-dependent degradation domain
OMI	inhibitors to X-linked inhibitor of apoptosis protein
PAMPs	Pathogen-associated molecular patterns
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PECAM-1	Platelet endothelial cell adhesion molecule-1
PHD	Prolyl hydroxylases
PIPs	Phosphatidylinositol phosphates
PMSF	Phenylmethylsulfonyl fluoride
PRRs	Pattern recognition receptors
pVHL	von Hippel-Lindau protein
RHD	Rel homology domain
RHIM	RIP homotypic interaction motif
RIG-I	Retinoic acid inducible gene 1
RIP	Receptor-interacting protein kinases
RIP1	Receptor interacting protein 1
RIP3	Receptor interacting protein 3
RLRs	Retinoic acid inducible gene I (RIG-I)-like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute media
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	Lupus erythematosus
SNPs	Single nucleotide polymorphisms
STAT3	Signal transducer and activator of transcription 3
TAE	Tris-acetate-EDTA

TAK1	Transforming growth factor-β (TGF-β) activated kinase
TAM	Tumour-associated macrophages
TBS	Tris-buffered saline
TBST	Tris-buffered saline, 0.1% Tween 20
TCR	T cell receptor
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TEN	Toxic epidermal necrolysis
Th1	T helper 1
TIF-2	Transcription intermediary factor 2
TIR	Toll/Interleukin-1 receptor
TIRAP	TIR-domain-containing adaptor protein (also MAL)
TLR	Toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNFR	Tumor necrosis factor receptor
TNFα	Tumor necrosis factor alpha
TPL2	Tumour progression locus 2
TRADD	TNF-R1-associated death domain protein
TRAF	TNF receptor-associated factor
TRAF6	TNF receptor associated 6
TRAIL	Tumor necrosis factor–related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TWEAK	TNF-related weak inducer of apoptosis
UPS	Ubiquitin proteasome system
VEGF	Vascular endothelial growth factor
WT	Wildtype
XIAP	X-linked inhibitor of apoptosis protein

CHAPTER ONE
GENERAL INTRODUCTION

1.1 The mammalian immune system

Immune systems are protective mechanisms present across species and manifest as basic enzymes in unicellular organisms, or phagocytosis, antimicrobial peptides and the complement system in plants and invertebrates, up to more sophisticated defences in vertebrates (Beck and Habicht, 1996; Litman, Cannon and Dishaw, 2005). The mammalian immune system is a complex ensemble of tissues, cells and processes responsible for defending an organism from infections agents. It is also the centre of homeostatic regulation keeping control over defective or malignant cells. There are several layers of protection in many species, including physical barriers which limit the entrance of bacteria and viruses to an organism, and then there are two more complex responses known as innate and adaptive immune systems (Litman, Cannon and Dishaw, 2005; Medzhitov, 2007; Kurosaki, Kometani and Ise, 2015). If a pathogen breaks the physical barriers (skin, internal epithelial layers or mucosal layers), the innate immune system provides a non-specific immediate response (Litman, Cannon and Dishaw, 2005). The adaptive immune system acts as a second layer of response providing a more specific pathogen recognition and a long-term protection in the form of an immunological memory (Restifo and Gattinoni, 2013; Kurosaki, Kometani and Ise, 2015). An adequate immunological response through both innate and adaptive immunity depends on an initial accurate distinction between self and non-self molecules (also known as antigens). Being the first line of response, the innate immune system has evolved to recognize a broad set of molecules that are conserved across microorganisms and also contains specific receptors for signals of damage, injury or stress in cells (Matzinger, 2002; Medzhitov, 2007). These receptors, called pattern recognition receptors (PRRs), can identify pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Medzhitov, 2007; Kumar, Kawai and Akira, 2011). The innate and adaptive responses interact in many ways. Adaptive response relies on soluble signals and on the phagocytosis and antigen presentation function of the innate response to initiate antigen specific clonal expansion in T and B lymphocytes (Iwasaki and Medzhitov, 2010). The adaptive response is sophisticated and highly specific; however, the innate response and its initial response and recognition of potential harmful signals is essential to trigger a proper immune response. Considering its crucial role, it is a highly regulated and widely complex system, and as part of this thesis some of those layers of regulation and interaction will be explored. For context, some of its main components and signalling pathways are initially described.

1.1.1 Pattern Recognition Receptors

There are different types of PRRs. The best known and well characterized PRRs are Nucleotide-binding oligomerization-like receptors (NLRs), RIG-I-like receptors (RLRs), cytosolic DNA-sensing receptors and Toll-like receptors (TLRs) (Kumar, Kawai and Akira, 2011; O'Neill, Golenbock and Bowie, 2013; Paludan and Bowie, 2013). NLRs are characterized by containing a C-terminal leucine rich repeat (LRR) domain that recognize microbial PAMPs or endogenous molecules, and a nucleotide-binding oligomerization (NACHT) domain required for activation of downstream signalling (Kumar, Kawai and Akira, 2011). Up to 23 human and 34 murine members of NLRs family have been described, with the NOD and inflammasome subgroups being the best-studied (Kumar, Kawai and Akira, 2011). The inflammasomes have been widely explored in the last years and are characterized as requiring a two-signal activation process. A first signal inducing the expression of Pro-Caspase 1, Pro-IL1β and Pro-IL18, and a second signal mediating inflammasome assembly and processing of these proteins (Palazon-Riquelme and Lopez-Castejon, 2018). Meanwhile, the RIG-I like receptors family consist of three members, RIG-I (Retinoic acid inducible gene 1), Mda-5 (Melanoma differentiation associated factor 5) and LGP2 (Laboratory of genetics and physiology 2). Their main characteristic is the DExD/H box RNA helicase domain which allows the identification of intracellular PAMPs in viral RNA (Kumar, Kawai and Akira, 2011). Finally, TLRs are the core of innate immune signalling and key regulators of the adaptive immune response (Manicassamy and Pulendran, 2009; Iwasaki and Medzhitov, 2010). They are the most relevant of the PRRs in the present study.

1.1.1.1 Toll-like receptors (TLRs).

TLRs are transmembrane glycoproteins similar in structure to Toll proteins, an important receptor in Drosophila embryogenesis. Ten genes in humans and 13 in mice have been identified for TLRs (name TLR1–13) (Akira and Takeda, 2004; Sato *et al.*, 2009; O'Neill, Golenbock and Bowie, 2013). TLRs can be found in the plasma membrane or inside endosomes, and each of them recognizes a specific PAMP or DAMP depending on their subcellular location (**Table 1.1 and Figure 1.1**). The functional heterodimers formed by TLR2/TLR1 and TLR2/TLR6, as well as TLR4, TLR5 and TLR11 are usually located on the cell membrane while TLR3, TLR7/TLR8, TLR9 and TLR13 are present inside endosomes (Akira and Takeda, 2004; Kumar, Kawai and Akira, 2011; O'Neill, Golenbock and Bowie, 2013). The main function of these receptors is to induce pro-inflammatory molecules but their role in cellular proliferation, survival, tissue repair and modulation of adaptive response have also been described (Iwasaki and Medzhitov, 2010; Li, Jiang and Tapping, 2010; Kawai and Akira, 2011).

Receptor	**DAMPs (endogenous)**	**PAMPs (exogenous)**	
	Ligand	**Ligand**	**Origin**
TLR1		Lipopeptides Soluble factors	Bacteria and Mycobacteria *Neisseria meningitidis*
TLR2	Heat-shock protein 60 (HSP60), HSP70 and HSP96 High-mobility group protein B1 (HMGB1) Hyaluronic acid	Lipoprotein/lipopeptides Peptidoglycan Lipoteichoic acid Lipoarabinomannan Phenol-soluble modulin Glycoinositolphospholipids Glycolipids Porins Atypical-lipopolysaccharide Zymosan	Various pathogens Gram-positive bacteria Gram-positive bacteria *Mycobacteria* *Staphylococcus epidermidis* *Trypanosoma cruzi* *Treponema maltophilum* *Neisseria* *Leptospira interrogans* and *Porphyromonas gingivalis* Fungi
TLR3	Double-stranded RNA (dsRNA), mRNA	Double-stranded RNA	Viruses
TLR4	HSP22, HSP60, HSP70, HSP96 HMGB1β-defensin 2 Extra domain A of fibronectin hyaluronic acid heparan sulfate fibrinogen surfactant-protein A	Lipopolysaccharide (LPS) HSP60 Envelope proteins Fusion protein Glycoinositolphospholipid Taxol	Gram-negative bacteria *Chlamydia pneumonia* Respiratory syncytial virus and mouse mammary tumor virus *Trypanosoma cruzi* Plant product
TLR5		Flagellin	Gram-positive or Gram-negative bacteria
TLR6		Diacyl lipopeptides Lipoteichoic acid Phenol-soluble modulin Zymosan Heat-liable soluble factor	*Mycoplasma* Gram-positive bacteria *Staphylococcus epidermidis* *Saccharomyces* Group B *streptococcus*
TLR7	Endogenous RNA	Single-stranded RNA (ssRNA)	Viruses
TLR8	Endogenous RNA	ssRNA	Viruses
TLR9	Endogenous DNA	Unmethylated CpG motifs Hemozoin	Bacteria and viruses Plasmodium
TLR10	Unknown	Unknown	
TLR11		Profilin	Uropathogenic bacteria
TLR12		Profilin	Uropathogenic bacteria
TLR13		Bacterial ribosomal RNA	*Toxoplasma gondii*

Table 1.1 Toll-like receptors (TLRs) and their DAMPs and PAMPs ligands. *DAMPs: Damage associated molecular pattern molecules; PAMPs: Pathogen-associated molecular patterns.* (Akira and Takeda, 2004; Sato *et al.*, 2009; Basith *et al.*, 2012; O'Neill, Golenbock and Bowie, 2013).

Considering their essential role in regulating the immune system, activation of TLRs signalling have also important roles in controlling tumor growth, progression and immune evasion (Huang *et al.*, 2005; Sato *et al.*, 2009; Multhoff, Molls and Radons, 2012).

The structural conformation of TLRs is defined by extracellular leucine rich repeats (LRRs) domain arranged in a horseshoe-like structure, which recognize PAMPs or DAMPs, a transmembrane domain and an intracellular Toll/Interleukin-1 receptor (TIR) domain (Akira and Takeda, 2004; O'Neill, Golenbock and Bowie, 2013). In mammals, TIR domain mediates TLR downstream signalling by recruiting four possible signalling adaptor proteins; Myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM), and MYD88-adaptor-like protein (MAL) (also known as TIR-domain-containing adaptor protein (TIRAP)) (**Figure 1.1**) (Akira and Takeda, 2004). These adaptors activate different patterns of gene expression depending on the TLRs that are being stimulated. MYD88 initiates the signalling cascade of most TLRs, except for TLR3 which depends exclusively on TRIF mediated activation. TLR5, TLR7/TLR8, TLR9, TLR11 and TLR13 all signal through MYD88 only, while TLR2/TLR1 - TLR2/TLR6 and TLR4 have been reported to use MAL and MYD88-dependent pathways. TLR4 can also be activated in a MYD88-independent way by interaction of the adaptors TRIF and TRAM (Akira and Takeda, 2004; O'Neill, Golenbock and Bowie, 2013). These adaptor proteins act as scaffolds for downstream signalling.

Activation of MYD88 by TLRs recruits and phosphorylates IL-1R-associated kinases (IRAK) 1, 2, 4 which in turn recruit and activates TAB2 and TNF- receptor-associated factor 6 (TRAF6). TRAF6 is an E3 ubiquitin ligase that interacts with the transforming growth factor-β (TGF-β) activated kinase (TAK1) complex containing TAK1 and TAK1-binding proteins (1 to 4) (O'Neill, Golenbock and Bowie, 2013). The activated TAK1 complex will mediate the signalling through the IκB kinase (IKK) complex, and the Mitogen Activated Protein Kinase (MAPK) such as P38 and c-Jun N-Terminal kinase (JNK) (Wang *et al.*, 2001). The IKK complex consists of two catalytic subunits known as IKK-α and IKK-β and a regulatory subunit, Nuclear Factor Kappa B Essential Modifier (NEMO). This complex phosphorylates the inhibitory IκB proteins which in their unphosphorylated states retain the Nuclear factor kappa-B (NF-κB) in an inactive form in the cytoplasm (Akira and Takeda, 2004). The phosphorylation of the IκB proteins target them for polyubiquitination and further proteasomal degradation, releasing NF-κB and allowing its translocation to the nucleus where it will activate a plethora of genes (Medzhitov, 2007). A similar mechanism of activation is used by TLR2/TLR1, TLR2/TLR6 and TLR4 with the additional initial interaction of the adaptor MAL with MYD88 that also leads to NF-κB and MAPKs activation mediated by IRAKs, TRAF6 and TAK1 complex (Kumar, Kawai and Akira, 2011).

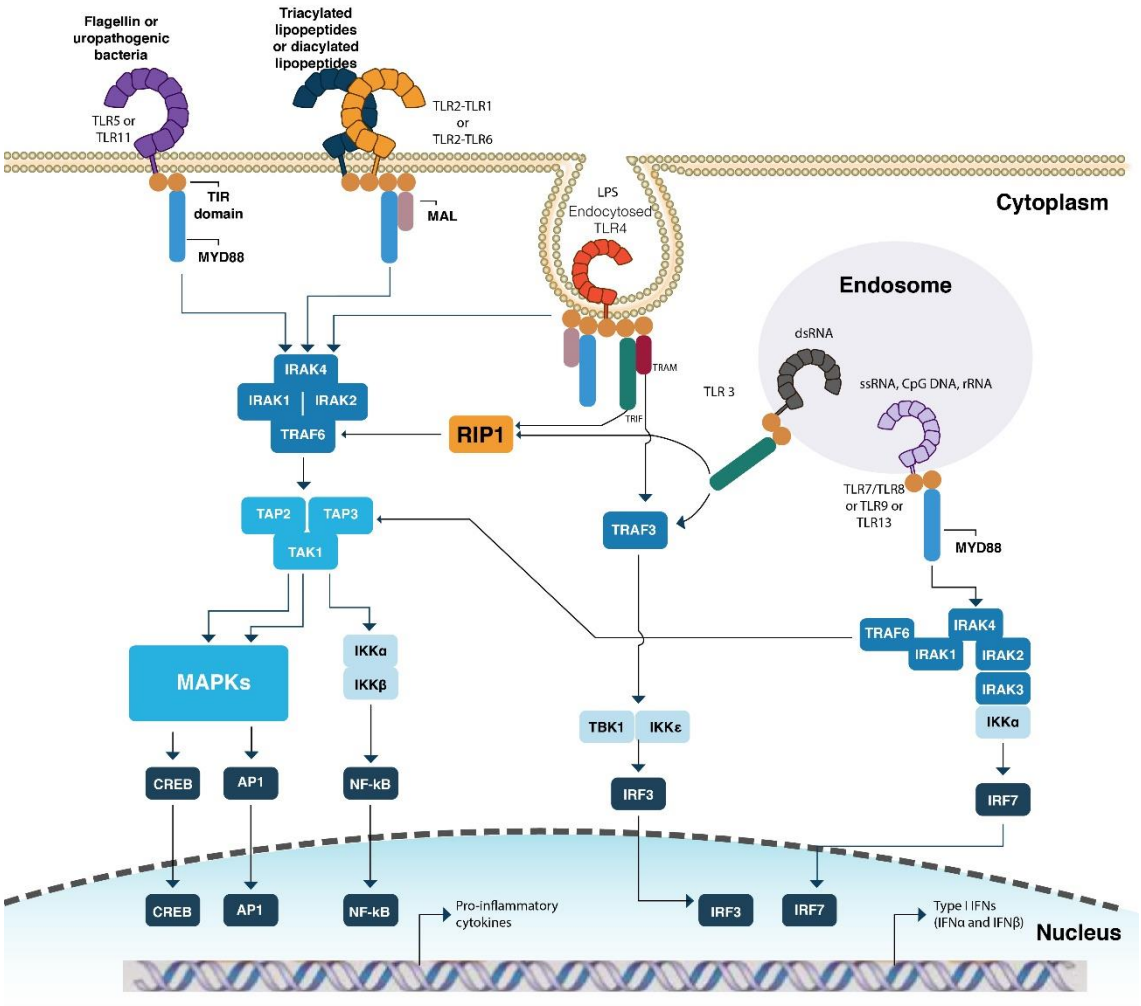

Figure 1.1 TLR signalling pathways.

From the 13 TLRs described in mice, TLR5, TLR11, and the heterodimers TLR2–TLR1 or TLR2–TLR6 are in the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 localize in endosomes. TLR4 is localized in the plasma membrane and it is later endocytosed. The signalling cascade initiated by TLRs begins with the ligand-induced dimerization of receptors and it is followed by the recruitment of adaptors proteins to the receptors TIR domains. Four TIR domain-containing adaptor proteins have been identified: MYD88, MAL, TRIF and TRAM. Cooperation and interaction between the TIR domains of the receptor and the adaptor proteins triggers a signalling cascade which includes IRAKs and TRAFs proteins. These proteins will in turn activate the corresponding transcription factor in a MAPKs, IKK or TBK1/IKKε-dependent way. NF-κB, IRF, CREB and AP1 are some of the transcription factors induced by TLRs engagement. TLR signalling

ultimately leads to production of pro-inflammatory cytokines and type I interferon (IFN). *dsRNA, double- stranded RNA; IKK, inhibitor of NF-κB kinase; IRAKs, IL-1R-associated kinases; LPS, lipopolysaccharide; MAL, MYD88-adaptor-like protein; MAPKs, mitogen-activated protein kinases; MYD88, myeloid differentiation primary-response protein 88; MKK, MAP kinase kinase; RIP1, receptor-interacting protein 1; rRNA, ribosomal RNA; ssRNA, single-stranded RNA; TAB, TAK1-binding protein; TAK, TGFβ-activated kinase; TIR, Toll–IL-1-resistence; TBK1, TANK-binding kinase 1; TRAFs, TNF receptor-associated factors; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor protein inducing IFNβ* (Adapted from O'Neill et al. 2013).

The activation of MYD88-independent pathways, characteristic of TLR3 and also possible with TLR4, is known to induce type 1 Interferons (IFNs) (Kumar, Kawai and Akira, 2011). TRIF, the adaptor mediating this type of signalling, recruits and activates the kinases TBK1 and IKKε through TRAF3 (Fitzgerald *et al.*, 2003; Saha and Cheng, 2006). Another player in this signalling pathway is the receptor interacting protein 1 (RIP1) which links TRIF signalling to NF-κB activation by interacting with TRAF6 (Sato *et al.*, 2003; Meylan *et al.*, 2004). Meanwhile, TLR4 requires the additional interaction with the adaptor TRAM to initiate its TRIF-dependent cascade of activation that will ultimately lead to the production of type 1 IFNs (Lee and Barton, 2014). All the TLR signalling pathways result in the secretion of pro-inflammatory or anti-inflammatory cytokines and chemokines, direct soluble effectors of the different types of immune response.

1.1.1.2 TNFα and TNFR1 signalling

Tumor necrosis factor α (TNFα) is one of the proinflammatory cytokines induced as a primary response to bacterial or viral challenge by TLR signalling (Parameswaran and Patial, 2010). TNFα is produced by monocytes/macrophages, activated NK and T cells, endothelial cells and fibroblasts as a transmembrane 26 kDa protein which will later be cleaved by a metalloprotease into a 17 kDa soluble TNFα (Sedger and McDermott, 2014). Transmembrane and soluble TNF can bind to two receptor molecules: the death-domain-containing TNFR1 or TNFR2. The binding of TNFα to its receptor TNFR1 has the potential to trigger the production of more proinflammatory cytokines but it can also influence cellular process like proliferation, differentiation or programmed cell death (Sedger and McDermott, 2014). Activation of TNFR1 receptor attracts TNF-R1 associated death domain protein (TRADD) proteins, FAS associated death domain protein (FADD), TRAF2 and RIP1 to the intracellular portion of this receptor (Hsu *et al.*, 1996). Depending on the intracellular molecules and conditions, this complex of proteins can trigger activation of NF-κB and MAPKs, processing of caspase 8 and subsequently initiation of apoptosis, or phosphorylation of RIP3 and activation of necroptosis (**Figure 1.2**) (Devin *et al.*, 2000; Cho *et al.*, 2009; Wilson, Dixit and Ashkenazi, 2009; Varfolomeev and Vucic, 2018). TNF signalling depends on linear ubiquitination of many of its associated molecules to regulate the activation of a specific signalling cascade. Linear polyubiquitination of RIP1 in the residues K11, K63 and K48 by E3 ligases cellular Inhibitor of Apoptosis 1 and 2 (c-IAP1 and 2) is one of the central regulatory mechanisms of TNFR1 activation (Devin *et al.*, 2000; Lee *et al.*, 2004; Bertrand *et al.*, 2008; Mahoney *et al.*, 2008; Varfolomeev *et al.*, 2008; Varfolomeev and Vucic, 2018). cIAPs are proteins constitutively bound to TRAF2 and therefore recruited to the intracellular TNFR1 complex after it has engaged the ligand TNFα (Devin *et al.*, 2000; Park, Yoon and Lee, 2004; Mahoney *et al.*, 2008). cIAPs1/2-mediated K63 ubiquitination of RIP1 in turn recruits

transforming growth factor b-activated kinase 1 (TAK1) through its ubiquitin-binding domain (Devin *et al.*, 2000; Chen, 2005). Other proteins like TAK1 binding protein 2 and 3 (TAB2 and 3), and complexes IκB kinase (IKK) and linear ubiquitin chain assembly complex (LUBAC) are also engaged (Sedger and McDermott, 2014). TAK1 phosphorylates the IKK complex, consisting of NF-κB essential modulator (NEMO) and IκB kinases 1 and 2 (IKK1/2), what allows NF-κB translocation to the nucleus and induction of pro-inflammatory genes (Chen, 2005; Mercurio *et al.*, 2018). E3 ligase HOIL-1 interacting protein (HOIP), haem-oxidized IRP2 ubiquitin ligase-1 (HOIL-1) and SHANK-Associated RH Domain Interactor (Sharpin) proteins have been described to form the LUBAC complex which was also described to mediate NF-κB activation in a RIP1-independent way (Haas *et al.*, 2009; Walczak, 2011). The LUBAC proteins SHARPIN and HOIL-1 can ubiquitinate some of the TNFR1-associated molecules, such as TRADD, RIP1 and NEMO, and in that way activate the IKK complex and NF-κB (Walczak, 2011; Varfolomeev and Vucic, 2018). Activation of TNFR1-mediated NF-κB signalling is blocked when RIP1 is deubiquitinated by the deubiquitinases cylindromatosis (CYLD) and A20 or in conditions favouring depletion of cIAP1/2 by Smac mimetics (Mahoney *et al.*, 2008; Declercq, Vanden Berghe and Vandenabeele, 2009; Wilson, Dixit and Ashkenazi, 2009). Under those conditions, TNFR1 signalling can be driven towards two types of programmed cell death by means of RIP1 activity. The activation of the apoptotic and necroptotic types of cell death will be discussed in detail in sections 3.1.1.1 and 3.1.1.2 of this thesis.

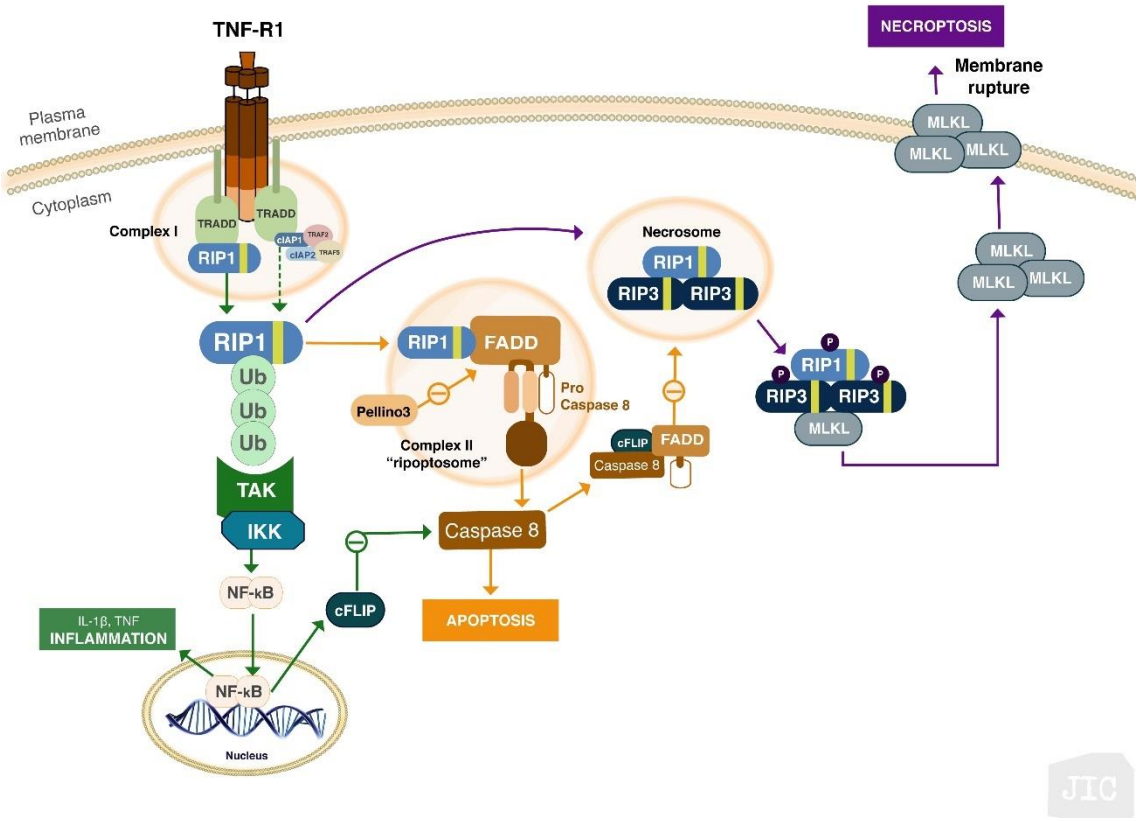

Figure 1.2 Signalling pathways activated by TNFR1 signalling.

Stimulation of cells with TNF can induce the activation of three signalling pathways. The first pathway is the inflammatory, where the binding of TNF ligand to its receptor induces the recruitment of TRADD, RIP1 and a number of E3 ligases (TRAF2, TRAF5, cIAP1 and cIAP2) to TNF-R1 intracellular domain. Ubiquitination of RIP1 by the E3 ligases leads to activation of NF-κB mediated by TAK1/IKK and consequently induction of pro-inflammatory signals and anti-apoptotic proteins like cFLIP. Under condition mediating RIP1 de-ubiquitination or in the absence of cIAP1 and cIAP2, RIP1 can interact with FADD and procaspase 8 to form the Complex II or "ripoptosome". Complex II facilitates caspase 8 auto-processing and triggers a downstream caspase cascade which ends in cell death by apoptosis. The E3 ligases Pellino 3 can block this signalling pathway by impeding Complex II formation. Finally, in absence of caspase 8, RIP1 and RIP3 can interact, autophosphorylate and assemble the necrosome. This structure allows the interaction of RIP3 with MLKL and subsequent phosphorylation that leads to the cellular membrane rupture and cell death by necroptosis. This last pathway can be inhibited by cleavage of RIP1 and RIP3 through the FADD/cFLIP/caspase 8 complex (Adapted from Moynagh et al. 2014).

1.1.2 Innate immune signalling cascades

The efficient and effective activation of an immune response depends on signalling cascades downstream of recognition of PAMPs, DAMPs or soluble molecules, engaging their corresponding receptors. The network of molecules involved in the signalling is extensive. However, the main effectors of TLR and TNF signalling are the pathways of the mitogen-activated protein kinase (MAPKs), and the signalling cascade leading to the activation of nuclear factors NF-κB and IRFs, which will be discussed next.

1.1.2.1 Mitogen-activated protein kinase (MAPKs)

Mitogen-activated protein kinases (MAPKs) are an extensive family of protein kinases that regulate essential cellular processes such as proliferation, differentiation, development and cell death in eukaryotic cells (Yang, Sharrocks and Whitmarsh, 2013). Three main subgroups have been identified and extensively studied: Extracellular Signal-Regulated Kinase (ERK), JNK/SAPK - P38 MAPK and ERK5 (**Figure 1.3**). Different stimuli activate a serial phosphorylation cascade of a set of three evolutionarily conserved kinases: a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK) (Cargnello and Roux, 2011). Each set of kinases activates a specific signalling cascade that interacts with many different downstream kinases or transcription factors. The one activating ERK1 and ERK2 induces differentiation, cell division and proliferation in response to growth factors and mitogenic stimulus (Cargnello and Roux, 2011; Yang, Sharrocks and Whitmarsh, 2013). The second subgroup includes two signalling pathways, one characterised by activation of P38 and one for JNK kinases. They are usually described together because they share most of their activators at MAPKKK level and both are phosphorylated in response to stress stimuli such as inflammatory cytokines, ultraviolet irradiation, oxidative stress, heat shock, or osmotic shock (Cargnello and Roux, 2011; Kyriakis and Avruch, 2012). These two are mainly involved in regulation of cellular processes such as differentiation, inflammation, cycle cell arrest and apoptosis (Yang, Sharrocks and Whitmarsh, 2013). Finally, ERK5 has been described to be initiated by developmental cues to guide endothelial formation, cardiac morphogenesis and brain development (Regan *et al.*, 2002; Glatz *et al.*, 2013).

1.1.2.2 NF-κB

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is one of the main nuclear factors to be activated in response to stimuli such as bacterial or viral antigens, stress, cytokines, free radicals, ultraviolet irradiation among others (Gilmore, 2006). Two pathways of activation have been described for NF-κB, known as Canonical or Classical and non-canonical or

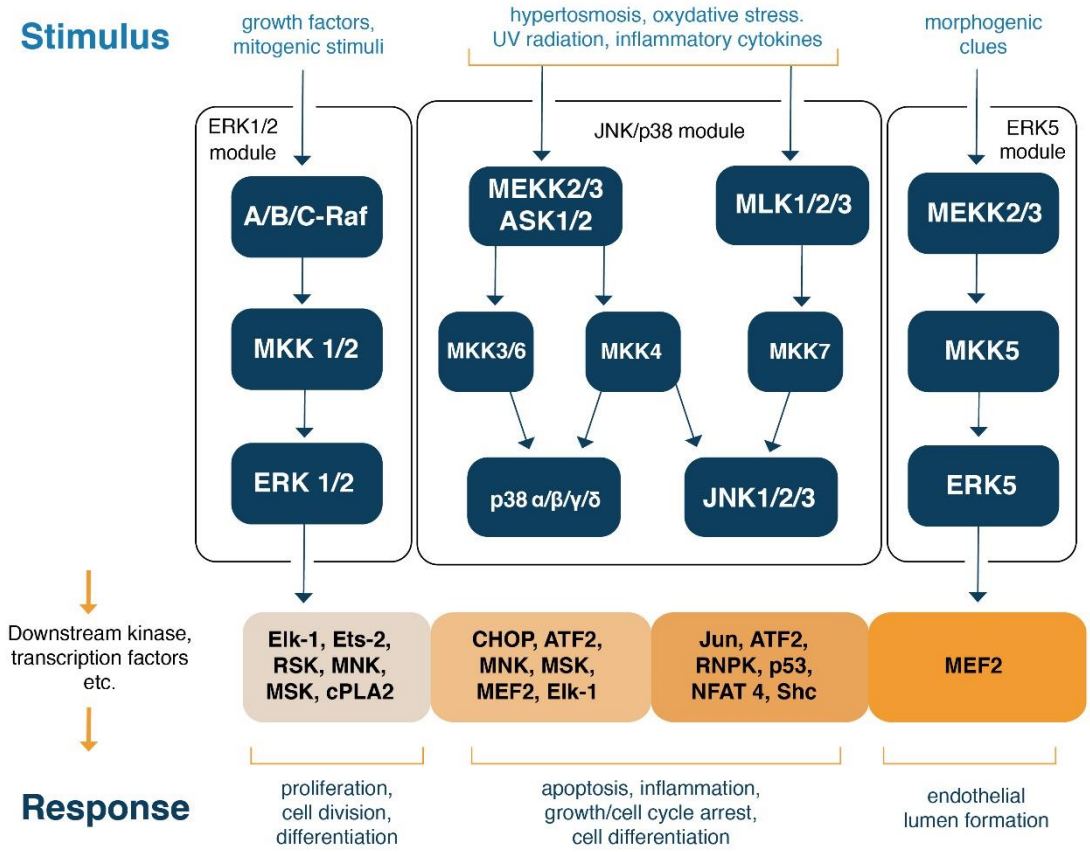

Figure 1.3 MAPKs signalling.

Extracellular Signal-Regulated Kinase (ERK), JNK/SAPK - P38 MAPK and ERK5 are the three main subgroups of MAPKs that have been described. They are activated by specific stimulus which trigger a serial phosphorylation cascade of conserved kinases that in turn regulate essential cellular processes such as proliferation, differentiation, development and cell death. Growth factors and mitogenic stimulus are known to induce ERK1 and ERK2 activation which main response is to induce differentiation, cell division and proliferation. Meanwhile, stress stimuli such as inflammatory cytokines, ultraviolet irradiation, oxidative stress, heat shock, or osmotic shock have been described to mediate the activation of P38 and JNK kinases that regulate cellular processes such as differentiation, inflammation, cycle cell arrest and apoptosis. Finally, developmental cues activate ERK5 which in turn guides endothelial formation, cardiac morphogenesis and brain development (Adapted from Yang, Sharrocks and Whitmarsh, 2013).

nonclassical pathways. The first one is initiated by most TRAF dependent signalling pathways and regulates cell survival and inflammation, while the second one depends on TNFRs signalling and it is associated to B-cell survival, adaptive immune response and development of lymphoid organ development (Gilmore, 2006). The family of NF-κB transcription factors includes five members which share an N-terminal Rel homology domain (RHD). This domain facilitates homo/heterodimerization and binding to target genes (Brasier, 2006; Gilmore, 2006). The five members of the family are p50/p105 (NF-κB1), p52/p100 (NF-κB2), p65, c-Rel and Rel-B. The last three contain a C-terminal transcription activation domain (TAD) in addition to RHD (Gilmore, 2006). NF-κB subunits are present in the cytoplasm as homo or heterodimers in an inactive form due to inhibition by IκB proteins (Karin and Ben-neriah, 2000; Brasier, 2006). The IκB proteins (IκBα, IκBβ and IκBε) contain multiple ankyrin repeats that mediate binding to NF-κB dimers, and their function is regulated by post-translational modifications such as phosphorylation and acetylation (Karin and Ben-neriah, 2000). All the IκBs are targeted for proteasomal degradation in response to pro-inflammatory signalling induced by TLRs or TNFα (Brasier, 2006). IκBs degradation unmask the nuclear localization signal (NLS) of NF-κB dimers and allows for their translocation to the nucleus where it binds to the promoter regions of genes encoding proinflammatory cytokines, cell adhesion molecules, growth factors, antiapoptotic proteins and other target genes (Brasier, 2006; Gilmore, 2006).

1.1.2.3 Interferon Regulatory Factors (IRFs)

The Interferon regulatory factors (IRFs) are another important family of transcription factors activated by PRRs signalling. They are known to mediate cellular process such as cell cycle, apoptosis and tumour suppression, but their main role is to the initiate anti-viral response by inducing the expression of type 1 interferons (IFN, IFN-α and IFN-β) (Honda, Takaoka and Taniguchi, 2006). Nine members of this family have been described, with IRF3 and IRF7 being the most broadly studied (Mamane *et al.*, 1999; Barnes, Field and Pitha-Rowe, 2003). The previously described proteins TBK1 and IKKε activate IRF3 and IRF7 by phosphorylation of their C-terminal serine residues (see section 1.1.1.1 TLRs) (Fitzgerald *et al.*, 2003). IRF3 activation is activated first, and it binds to the IFN promoter inducing production of IFN-β which in turn activates the IFN pathway and induction of IRF7 expression (Moynagh, 2005).

1.1.3 Regulation of innate immune signalling pathways

Immune responses are a complex network of interactions between proteins of different signalling pathways. Considering the potential negative impact on cell survival or tissue integrity as well as the energetic cost of activating the production of soluble proteins or initiating adaptive responses, it is understandably highly regulated. There are several layers to the regulation of the immune responses with post-translational modification being one of the most crucial ways of control (Liu, Qian and Cao, 2016). Modifications include phosphorylation, ubiquitination and SUMOylation and they play various roles ranging from regulating formation of signalling complexes to compartmentalization or protein trafficking (Liu, Qian and Cao, 2016). Once the receptor recognizes a potential harmful ligand and the corresponding signalling cascades are activated by post-translational modification, a particular response is then initiated. Proliferation, differentiation, inflammation, cell death or metabolic modifications represent some of the functional consequences of these events. The early studies in this thesis focus on the role of ubiquitination in innate immune signalling pathways.

1.1.3.1 Regulation of innate immune signalling pathways by ubiquitination

Post-transcriptional modification of proteins by ubiquitination or deubiquitylation regulate a broad set of immunological responses, from cell differentiation, antigen processing to inflammation (Komander and Rape, 2012; Liu, Qian and Cao, 2016). Ubiquitination of proteins is frequently associated with covalent attachment of polyubiquitin chains with each ubiquitin protein being attached to the preceding one by an isopeptide bond between the C-terminal glycine residue and the ε amino group of one of 7 lysine residues (K6, K11, K27, K29, K33, K48 or K63) of the preceding ubiquitin or the N-terminus of a second ubiquitin in a methionine residue (M1) (Komander and Rape, 2012; Swatek and Komander, 2016). The variety of polyubiquitin chains and the specific site of binding of subsequent ubiquitin governs the fate of the targeted proteins (Ravid and Hochstrasser, 2008; Komander and Rape, 2012; Swatek and Komander, 2016). Furthermore, recent research has pointed other possible post-translational modifications on ubiquitin chains such as SUMOylation, phosphorylation and acetylation, which adds extra layers of complexity to the ubiquitin code (Swatek and Komander, 2016). Proteins ubiquitination was for long time defined as a signal for proteasomal degradation. However, more recent research has proven it also has roles in non-degradative processes labelling proteins for signalling purposes such as endocytosis or scaffolding and regulating a plethora of biological process including cell cycle, epigenetic, protein trafficking, mitophagy, response to DNA damage and NF-κB signalling among others (Komander and Rape, 2012; Swatek and Komander, 2016).

The most well characterised ubiquitination linkages are via lysine 48 (K48) or (K63). K63-linked polyubiquitination mediates TLR signalling and the production of type I IFNs by regulating the activity of TRAF6, TAB2/3, NEMO, TRAF3, RIG-I, MAVS and STING (Liu, Qian and Cao, 2016). K63-linked polyubiquitination of RIP2 by Pellino 2 has also been proved to be essential in NOD2 signalling (Yang, Wang, Humphries, *et al.*, 2013). In addition, ubiquitination of ASC by LUBAC and deubiquitination of NLRP3 are required for the activation of the NLRP3 inflammasome (Broz and Dixit, 2016). K48-linked polyubiquitination is associated with tagging of proteins for proteasomal degradation with IκB degradation being an exemplar of this process (Chen, 2005; Brasier, 2006; Gilmore, 2006; Mercurio *et al.*, 2018). The proteasome mediates degradation of K48-linked ubiquitinated proteins. The proteasome can be blocked by pharmacological agents such as MG132 with some inhibitors such as Bortezomib being used clinically to treat cancers such as multiple myeloma. These inhibitors are used in the present study to further characterise the role of the proteasome in control of the anti-inflammatory pathways.

Over the last years, research has indicated other types of polyubiquitination linkages through K6, K11, K27, K29 or K33 to play important roles in immune signalling and inflammation (Heaton, Borg and Dixit, 2016; Liu, Qian and Cao, 2016). Protein ubiquitination relies on the sequential activation of 3 types of enzymes (Deshaies and Joazeiro, 2009; Komander and Rape, 2012; Inobe and Matouschek, 2014). They include a ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3)(Komander and Rape, 2012; Metzger *et al.*, 2014). Part of this thesis will focus on a particular family of E3 ubiquitin ligases known as Pellino proteins and their role in innate immune signalling with particular focus on regulation of cell death. There are three members of this family containing common RING and forkhead-associated (FHA) domains (Lin *et al.*, 2008; Moynagh, 2014). The E3 Pellino family have been linked to mediate the ubiquitination of several proteins in a variety of signalling pathways including TLR, NOD and TNF signalling (Moynagh, 2014). They are closely associated with RIP kinases at the molecular and functional level and for this reason are explored in this thesis in the context of cell death.

1.1.3.2 Cell death and inflammation

Cell death plays important roles in regulation of inflammation and innate immunity (Kolb *et al.*, 2017). Cell death was first seen as an accidental process occurring when cellular functions were beyond repair. However, an extensive amount of research has described different types of regulated cell death including apoptosis, necroptosis (a regulated subtype of necrosis), autophagy and pyroptosis (Galluzzi *et al.*, 2014; Kolb *et al.*, 2017). Apoptosis and autophagy are

regarded as anti-inflammatory types of cell death while necroptosis and pyroptosis have been suggested to be pro-inflammatory (Taylor, Cullen and Martin, 2008; Kaczmarek, Vandenabeele and Krysko, 2013; Pasparakis and Vandenabeele, 2015; Kolb *et al.*, 2017). However, some researchers have proposed necroptosis to also be anti-inflammatory (Kaczmarek, Vandenabeele and Krysko, 2013; Kearney *et al.*, 2015). Apoptosis and necroptosis molecular pathways are closely related in terms of triggers, mediators and key molecular regulators (Humphries *et al.*, 2014; Huang *et al.*, 2015; Vasudevan and Ryoo, 2015). The family of receptor interacting protein (RIP) kinases are key determinants in the types of cell death (Moriwaki and Chan, 2013; Humphries *et al.*, 2014). As previously described in Section 1.1.3.1, post-translational modifications are essential for the regulation of RIP kinases activity, particularly ubiquitination mediated by E3 ligases like the Pellino family (Humphries *et al.*, 2014; Moynagh, 2014). This will be further characterised in this thesis.

1.1.3.3 Hypoxia and innate immune pathways

Ubiquitination plays important roles in regulation of transcription factors like NF-κB and cell death pathways in innate immune signalling. It is also critically important in responding to conditions of low oxygen (hypoxia) an environment that is typically found at sites of inflammation. Hypoxia regulates the activation of multiple genes associated with essential cellular process such as cell migration, metabolism, signalling and cell fate (Iyer, Leung and Semenza, 1998; Lisy and Peet, 2008). The cellular responses to hypoxia are coordinated by a group of proteins known as hypoxia-inducible transcription factors (HIFs) that under normal oxygen tension are degraded by the ubiquitin proteasome system (Lisy and Peet, 2008). Inflammation and tissue hypoxia have been correlated in several diseases from inflammatory bowel disease (IBD) to obesity (Colgan and Taylor, 2010; Cummins *et al.*, 2016). Its upregulation has also been extensively discussed as a mechanism of survival for malignant cells (Campbell *et al.*, 2014; Campbell and Colgan, 2015; Cummins *et al.*, 2016). HIF signalling has been associated with multiple immune pathways including TLR3 and TLR4 signalling, and its molecular functions are also linked with transcription factors such as NF-κB, STAT3 and AP-1 (Noman *et al.*, 2009; Oblak and Jerala, 2011; Bruning *et al.*, 2012; Tewari *et al.*, 2012; Pawlus, Wang and Hu, 2014; Veyrat *et al.*, 2016; Matijevic Glavan *et al.*, 2017). In addition, research from our group has described interaction between TRAF6, HIF-1α and IL1β as a mechanism mediating insulin resistance in a murine model of obesity (Yang *et al.*, 2014). In this thesis, we explore the interface of innate immune signalling and activation of HIF.

1.2 Project aims

1.2.1 Overarching Objective

Innate immune signalling is crucially important in triggering activation of transcription factors such as NF-κB and HIF that regulate expression of genes encoding pro-inflammatory and anti-inflammatory cytokines. However, there is also an emerging appreciation of the important contribution of necrotic forms of cell death to inflammation. The ubiquitin/proteasome system (UPS) is a key regulator of all of these processes. The overarching theme of this study is revise the roles of the ubiquitination and proteasome systems in innate immune signalling with particular focus on cell death, HIF and control of expression of the anti-inflammatory cytokine IL10.

1.2.2 Specific aims

- Explore the role of Pellino 2 and Pellino 3 E3 ubiquitin ligases in the regulation of the programmed type of cell death known as necroptosis.
- Characterise of the ability of TLRs to trigger HIF-1α signalling.
- Delineate the mechanism underlying the interplay between the proteasome and expression of the anti-inflammatory cytokine IL10.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

Reagent	Supplier
2-DOG	Sigma
Absolute Ethanol	VWR prolabo chemicals
AEG3482	Tocris
Agarose	Sigma
AMG548	Tocris
Amlexanox	Invivogen
APS	Sigma
ATP	Sigma
Bioscrip Reaction Buffer (5X)	Bioline
Bioscrip Reverse transcriptase	Bioscience
Birinapant	APExBIO
Bortezomib (PS-341)	Selleckchem
Bradford Reagent	Sigma
Bromophenol blue	Sigma
BSA	Sigma
BV6	SelleckChem
Chloroform	Sigma
Chloroquine	Invivogen
Complete mini protease Inhibitor cocktail	Roche
CpG ODN 1668	Invivogen
Cycloheximide (CHX)	Sigma
DirectPCR (tail)	Viagen
DMEM	Fisher
DMOG	Enzo
DMSO	Sigma
dNTPs (deoxyribonucleotide triphosphates)	BioLabs
DTT	Sigma
EDTA (Ethylenediaminetetraacetic acid)	Sigma
Ethanol	Sigma
FBS	Thermo fisher
GelRed Nucleic acid stain	Biotium
Glacial acetic acid	Sigma
Glycerol	Sigma
Glycine	Fisher
GoTaq®Green Master Mix	Promega
Hydrochloric acid (HCL)	Merk
Igepal	Sigma
IRAK1-4 inhibitor	Millipore
Isopropanol	Fisher
LCL161	Selleckchem

LPS	Enzo
Methanol	Sigma
MG132	Selleckchem
Na3VO4	Sigma
NaCl	Sigma
NaF	Sigma
Necrostatin-1	Sigma
NewBlotTM Nitro Stripping buffer	Licor Biosciences
NF-κB SN50	Enzo
PBS	Sigma
Pellino 3 SiRNA	Life tech
Penicillin Streptomycin	Gibco
PMSF	Sigma
Poly(I:C)	Invivogen
Protease Inhibitor Cocktail	Roche
Protein A/G beads	Santa Cruz
Protein K	Qiagen
Protein ladder	Fisher
Protogel	National diagnostics
Random primers	Invitrogen
Rapamycin	Invivogen
RIPK inhibitors (GSK'872)	GSK
RPMI	Fisher
SDS	Sigma
Skim milk powder	Tesco
Sodium chloride (NaCl)	Sigma
Sodium hydroxide (NaOH)	Sigma
Sodium orthovanadate (Na_3VO_4)	Sigma
Sodium Phosphate (Na_3PO_4)	Sigma
Sucrose	Sigma
Sulfuric acid (H_2SO_4)	Sigma
Taq polymerase	Invitrogen
TEMED	Sigma
TMB ultra sensitive substrate	Moss substrates
TNFα	R&D Systems
Trizma - Tris	Sigma
Trizol	Ambion
Trypsin EDTA free	Gibco
Trypsin-EDTA solution	Sigma
Tween 20	Sigma
WesternBright ECL Substrate	Advansta
X-Ray Developer	RG
X-Ray Fixer	RG
zVAD-fmk	Enzo
β-mercaptoethanol (BME)	Thermo-Fisher

2.1.2 Kits

Kits	Supplier
FITC Annexin V Apoptosis Detection kit	BD Pharmingen™
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega
Mouse CXCL2/MIP-2 DuoSet ELISA	R&D Systems
Mouse IL-10 ELISA	R&D Systems
Mouse IL-6 ELISA	R&D Systems
Mouse KC /CXCL1	R&D Systems
Mouse TNFα ELISA	R&D Systems

2.1.3 Antibodies

Antibodies	Supplier
Primary	
Caspase-3	Santa Cruz
Cleaved caspase-8	Cell Signalling
Cleaved PARP	Cell Signalling
CREB (86B10)	Cell Signalling
FLIP	Cell Signalling
GAPDH	Cell Signalling
HIF-1α	Novusbio
p38 MAPK (Thr180/Tyr182)	Cell Signalling
P44/42 MAPK (Erk1/2)	Cell Signalling
P-CREB	Cell Signalling
Phospho-MLKL	Abcam
Phospho-p38 MAPK (Thr180/Tyr182) (D3F9)	Cell Signalling
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signalling
Phospho-SAPK/JNK (Thr183/Tyr185)	Cell Signalling
Phospho-Stat3 (Tyr705)	Cell Signalling
P-Ikkαβ	Cell Signalling
P-Ikβα	Cell Signalling
P-NF-κB p65	Cell Signalling
P-TBK1	Cell Signalling
RIP1	BD
RIP3	Cell Signalling
SAPK/JNK	Cell Signalling
β-actin	Sigma
Secondary	
IRDye 800CW Goat Anti-Rabbit	Licor Biosciences
IRDye 680 Goat Anti-Mouse	Licor Biosciences
IRDye 800CW Donkey Anti-goat	Rockland
Anti-mouse / rabbit-HRP	Cell Signalling

2.1.4 Primers

Gene	Sequence
Sigma-Aldrich	
HPRT	FM1: 5'AGGGATTTGAATCACGTTTG RM1: 5'TTTACTGGCAACATCAACAG
CXCL10	FM1: 5'AAAAAGGTCTAAAAGGGCTC RM1: 5'AATTAGGACTAGCCATCCAC
GLUT-1 (Slc2a1)	FM1: 5'AAGTCCAGGAGGATATTCAG RM1: 5'CTACAGTGTGGAGATAGGAG
VEGF	FM1: 5'TAGAGTACATCTTCAAGCCG RM1: 5'TCTTTCTTTGGTCTGCATTC
IL10	FM1: 5'CAGGACTTTAAGGGTTACTTG RM1: 5'ATTTTCACAGGGGAGAAATC
Genotyping for Pellino 3 KO	2092_25: CCCAACATAGGTGTTTCCTCTCC 2092_29: GTGCATACACATTCATGCAAGC 2091_27: GACACGTGTGGAGATAATGAGG 2091_28: ACCCAGGCACAAGTCAAGC
Genotyping for Pellino 2 KO	4892_127: GCCTCTACAGGATGCTCATTT 4892_128: GGACAGTCATGCTAGTCTGAGG 4893_125: GAGACTCTGGCTACTCATCC 4893_129: CCTTCAGCAAGAGCTGGGGAC
Control Primers	1260_1: GAGACTCTGGCTACTCATCC 1260_2: CCTTCAGCAAGAGCTGGGGAC

2.1.5 Buffers

Buffers	Composition
Blocking buffer	5% Non-fat dry milk in Tris buffered saline plus Tween 20 (TBST)
Cell lysis Buffer	20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% (v/v) Igepal, 10% (w/v) glycerol, 50 mM NaF,1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, complete protease inhibitor cocktail (Roche, mini)
Laemmli sample buffer Loading buffer (1X)	62.5 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, 0.7 M β-mercaptoethanol, 0.001% (w/v) bromophenol blue
Lower Tris buffer (4X)	1.5M Tris, 0.4% SDS, dH_2O pH 8.8

PBS	2.7mM KCl, 1.5mM KH_2PO_4, 137mM NaCl, 8mM Na_2HPO_4, pH 7.4
Reagent diluent for ELISA	0.1% (w/v) BSA, 0.05% (v/v) Tween in TBS
SDS Running buffer (10X)	0.25M Tris, 1.92M glycine, 1% SDS, dH_2O
TAE (Tris-acetate-EDTA) buffer	40 mM Tris base, 0.1% (v/v) glacial acetic acid, 1 mM EDTA
Transfer buffer	250 mM Tris, 192 mM glycine, 15%/20% methanol, dH_2O
Tris buffered saline (TBS)	25mM Tris, pH7.4, containing 0.14M NaCl.
Tris buffered saline (TBST)	Tris-buffered saline, 0.1% Tween 20
Upper Tris buffer (4X)	0.5M Tris, 0.4% SDS, dH_2O pH 6.8

2.1.6 Cells

Cells	**Description**
iBMDMs WT	WT Immortalized Bone Marrow Derived Macrophages
iBMDMs $TRIF^{-/-}$	$TRIF^{-/-}$ Immortalized Bone Marrow Derived Macrophages
iBMDMs $IRF3^{-/-}$	$IRF3^{-/-}$ Immortalized Bone Marrow Derived Macrophages
L929	Mouse fibroblasts
THP-1	Human monocytes

Immortalized BMDMs cell lines were generated by infection with J2 recombinant retrovirus (Roberson and Walker, 1988). Macrophage cell lines from wild-type (C57BL6), $TRIF^{-/-}$ and $IRF3^{-/-}$ mice were generated and are referred to as iBMDMs WT, iBMDMs $TRIF^{-/-}$ and iBMDMs $IRF3^{-/-}$. Primary BMDMs were incubated in L929 conditioned medium for 3–4 days to induce macrophage differentiation (See Section 2.2.1.2). Cells were then infected with J2 recombinant retrovirus and maintained in culture for 3–6 months slowly weaning off the percentage of L929 conditional medium until cells were growing in its absence. Macrophage phenotype was confirmed by surface marker expression for CD11b and F480 and functional parameters such as responsiveness to TLR ligands and bacterial uptake.

L929 (85011425-1VL) and THP-1 (88081201-1VL) cell lines were acquired from Sigma.

2.1.7 Animals

Pellino 2 and Pellino 3 deficient mice were generated by Taconic Artemis using RNAi-modified transgenic mice proprietary technology which allows for the development of custom-engineered knockout mouse models. This technology is based on the development of a target vector where the gene of interest is silenced. The vector is then sequenced and electroplated into C57BL/6NTac ES cells. Clones are validated via Southern Blot and subsequently injected into mouse blastocysts for transfer to pseudopregnant females. First generation clones are bred to delete mice and remove selection markers until second generation knockout mice are determined to be a competent germline. After their development by Taconic Artemis, Pellino 2 and Pellino 3 deficient mice and their wild-type counterparts were bred at the animal facility at Biosciences, Maynooth University. Genotyping was performed by PCR analysis of genomic DNA from ear punches as described in 2.2.1.4 and genotypes were reconfirmed after the completion of experiments. The mice were housed in standard rodent cages, 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 Health Products Regulatory Authority (HPRA), and all procedures were approved by the research ethics committee of the Maynooth University. Double knockout *Peli2*$^{-/-}$*Peli3*$^{-/-}$ mice were generated by crossing *Peli2*$^{-/-}$ and *Peli3*$^{-/-}$. Genotyping was performed as described in section 2.2.1.4 by performing three independent reactions for the corresponding alleles in the same sample (See Section 2.1.4).

2.1.8 Gifts

Immortalized BMDMs WT, TRIF$^{-/-}$ and IRF3$^{-/-}$ were kindly provided by Prof. Kate Fitzgerald at (University of Massachusetts Medical School).

2.2 Methods

2.2.1 Cell culture and sample preparation

2.2.1.1 Cell culture

L929 and THP-1 cells were cultured in RPMI medium supplemented with 10% (v/v) fetal bovine serum (FBS, Thermo fisher), 100 µg/mL of Penicillin Streptomycin (Penstrep, Gibco). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and passaged every 2 to 3 days by scraping and subsequent centrifugation. Cells were then resuspended in new medium and seeded for experiments or for expansion of the cell cultures. Conditional M-CSF media: L929 were seeded at a confluency of $5x10^5$ cells/mL in 40 mL RPMI in a T175 flask and incubated for 7 days at 37°C in a humidified atmosphere of 5% CO_2. After 7 days, supernatant was transferred to a 50mL tube and centrifuged at 600 g for 5 min to remove dead or floating cells. Cells were cultured for a maximum of 25 passages to avoid excessive genetic drift. Supernatants were collected and stored at -80°C until use.

Immortalized cell lines for WT, $TRIF^{-/-}$ and $IRF3^{-/-}$ BMDMs were also used and cultured as follows: cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS, Thermo fisher), 100 µg/mL of Penicillin Streptomycin (Penstrep, Gibco). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and passaged every 2 to 3 days by scraping. Cells were then resuspended in new medium and seeded for experiments or for expansion of the cell cultures.

2.2.1.2 Bone-Marrow Derived Macrophages (BMDMs)

Bone marrow was flushed from tibia and femur of *Peli3*$^{+/+}$, *Peli2*$^{+/+}$, *Peli2*$^{-/-}$ and *Peli3*$^{-/-}$ mice. BMDMs were differentiated by culture of bone marrow cells in RPMI medium supplemented with FBS, Penstrep and 10% conditioned medium of L929 mouse fibroblasts (macrophage-colony stimulating factor (M-CSF); 20 ng/ml) for 5 to 7 days.

2.2.1.3 Murine Embryonic Fibroblast (MEFs)

Thirteen or fourteen days embryos from breeding of Pellino 3 heterozygous (*Peli3*$^{+/-}$) mice were obtained from pregnant female mice. Females were euthanized by cervical dislocation according to standard protocols. The uterus was removed under sterile conditions and embryos transferred separately into Petri dishes with sterile PBS. The head of each embryo was separated

and processed for genotyping, and the red tissue (heart and liver) cut off to obtain the carcasses. Embryo carcasses were transferred to 50 mL tubes with 4 ml trypsin-EDTA solution, macerated and incubated at 37 °C for 15 min. Maceration was repeated twice, each time with a 10 min incubation at 37°C. DMEM (10% FBS and Penstrep) (12 mL) was added to inactivate trypsin and samples were pipetted up and down several times. Clumps of tissue were removed and media containing cells transferred into T75 tissue culture flasks, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. *Peli3*$^{+/+}$, *Peli2*$^{+/+}$, *Peli2*$^{-/-}$ and *Peli3*$^{-/-}$ MEFs were selected after genotyping and passaged every 3 to 4 days using Trypsin-EDTA solution. MEFs reach crisis phase around passage 10 and proliferation decreases at that stage precluding their further use. For genotyping embryo's heads were digested in 100µl Direct PCR Lysis Reagent (Tail) (Viagen Biotech) with 0.5 µl Proteinase K (QIAGEN). Samples were incubated at 55°C for at least 3 hrs or overnight. Subsequently, digested tissue was incubated at 85°C for 45mins to inactivate Proteinase K and diluted 1:10 in distilled water for PCR processing.

2.2.1.4 PCR-based genotyping

PCR amplification was performed on genomic DNA using GotoTaq DNA polymerase and specific primers to detect heterozygous/homozygous conventional alleles (See Section 2.1.4).
Reaction:

Template	2 µl
5X Buffer	5 µl
$MgCl_2$ (25mM)	2 µl
Primers (4pmol/µl)	2.5 µl each
dNTP mix (10mM each)	1 µl
GotoTaq (5u/µl)	0.15 µl
PCR-grade water	to 25 µl

For each target the samples were initially heated to 94°C for 5 min. This was followed by 35 cycles at 95°C for 30s, at 60°C for 30s and at 72°C for 1 min. Samples were then incubated at 72°C for 10 min and stored at 4°C. The PCR products were subjected to electrophoresis on a 2% (w/v) TAE agarose gel containing 5µg/ml GelRed Nucleic acid stain (Biotium) at 120 Volts for 20 minutes. Nucleic acids were visualized under ultraviolet light (UV) (254nm) and images were acquired using the SynGene Gbox gel documentation system (Frederick, MD, USA).

2.2.1.5 Concentration of the inhibitors

The concentration for the different inhibitors was selected based on the corresponding Datasheet, the literature or optimization experiments performed in the laboratory. Concentration for IAPs inhibitors (BV6, LCL161 and Birinapant), used in Chapter Three of this thesis, were determined by Dr. Fiachra Humphries using dose-dependency experiments. Concentrations for inhibitors used in Chapter Four (MG132, DMOG, Chloroquine, 2-DOG) were selected based on literature review and information from the providers. Concentration of Bortezomib was evaluated by dose-dependency experiments as described in section 5.2.3.

2.2.1.6 Isolation of proteins for Western Blot

Cells were plated in 12-well plates at a density of $1.0x10^6$/ml and treated as indicated. Depending on the type of study, harvest was carried as follows: (A) for cell death analysis, cells were scraped and transferred to 1.5mL microcentrifuge tubes. They were then centrifuged at 6000g for 1 min. Supernatants were retained and cells washed in ice-cold PBS, followed by 1 min centrifugation at 6000g. PBS was discarded and 80uL of sample buffer added. Samples were incubated 20min at 100°C and stored at -20°C. (B) for hypoxia analysis, media was discarded. Cells were washed once with cold PBS, and 100µL sample buffer added to each well. Cells were scraped into the sample buffer and transferred into new Eppendorf tubes to be heated at 95°C for 10 minutes. Samples were stored at -20°C until used.

2.2.1.7 Isolation of total RNA

Cells were seeded at a concentration of $1.0x10^6$ in 12-well plates. RNA isolation was carried out under RNase-free conditions to avoid introducing RNase contamination. For adherent cells, growth media was removed, and cells washed with 0.5mL of ice-cold PBS. TRIzol® Reagent (0.5 mL) was added directly to the cells in a 12-well plate and allowed to lyse by slowly shaking at room temperature for approximately 2 minutes. TRIzol containing the RNA was then transferred into new Eppendorf tubes and stored at -20°C or used immediately for RNA isolation which was done as per manufacturer's protocol. Briefly: 100 µL of chloroform were added to tubes containing the samples in TRIzol, the mixture was then vortexed for 15 sec and incubated at RT for 3 minutes. After incubation, samples are centrifuged at 12,000 g for 15 minutes at 4°C. Approximately 120 µL of the colourless-RNA upper aqueous phase was transferred to a new sterile tube and 250 µL of isopropanol added to the aqueous phase and subsequently vortexed for at least 5 sec. Samples were incubated at RT for 10 minutes, followed by centrifugation at 12,000 g for 10 minutes at 4°C. The isopropanol was carefully removed without disturbing the

precipitated RNA pellet and 1mL of 75% (v/v) ethanol were added. Samples were vortexed briefly and centrifuged at 7,500g for 5 min at 4°C. Ethanol was removed and the RNA pellets were air dried on a heating block at 60°C for up to 10 min. The RNA pellet was re-suspended in 30µL of RNase free water and incubated at 65°C for 10 minutes. Concentrations and purities of isolated RNA were determined by means of a Nanodrop™ Spectrophotometer using absorbance at 260 and 280nm wavelengths. RNA was stored at -20°C.

2.2.1.8 Synthesis of first strand cDNA from messenger RNA (mRNA)

cDNA was produced from total RNA extracted as previously described. RNA (1 µg) was diluted in nuclease free water to a final volume of 11.5 µl. Random primers (0.5 µl) were added and the mix incubated at 70°C for 5 min. Next, the mixture was allowed to cool down on ice before adding the following components:

Reagent	**Vol.**
Bioscript reverse transcriptase (200U/µl)	0.5µl
dNTPs (10mM)	1µl
5 x Bioscript Reaction Buffer	4µl
H_2O	2.5 µl

The reaction mixture was incubated at 42°C for 1h, heated at 70°C for 10mins to deactivate Bioscript reverse transcriptase, and cooled at 4°C for 5mins. cDNA was then stored at -20°C.

2.2.1.9 Real –time quantitative PCR

cDNA samples were diluted 1:100 by addition of PCR-grade water. The real-time PCR reaction mixture was prepared in 96-well plates as follows to a final volume of 20µl:

Reagent	**Vol.**
Master Mix (2x) SensiMix SYBR No-ROX	10 µl
Primers (4pmol/µl)	2,5 µl each
PCR-grade water	Up to 20 µl (3µl)
Samples	2 µl each well

Samples were assayed in duplicate and normalised relative to levels of a housekeeping gene (HPRT). Primer sequences were as described in Materials. Once the mix was set up, the cyclical

RT-PCR reactions were performed in an Applied Biosystems Step One PLUS real-time instrument as follows: denaturation was carried at 95°C for 15 minutes, followed by 40 cycles at 95°C for 30 seconds, 57°C for 30 seconds (according to primer) and 72°C for 30 seconds. Integration of the fluorescent SYBR Green into the PCR product was monitored after each annealing step. Amplification of one specific product was confirmed by melt curve analysis where a single melting peak eliminated the possibility of primer-dimer association. For melting curve analysis to be performed the products were heated from 60°C to 95°C after the 40 cycles. The PCR reactions were conducted in a Bio-Rad ICycler realtime PCR instrument. Targeted gene expression activity was determined as relative quantification using the Cycle Threshold (CT) method. CT represents the number of cycles required to detect a fluorescent signal, corresponding to the target amplicons, intersecting a preprogramed fluorescence threshold line. The concentration of target nuclei acid is inversely proportional to CT values, the higher the concentration of target the less cycles are necessary to detect the signal. The variations in the target nuclei acid (ΔCT) value is calculated by subtracting the CT value of a control (for this thesis the control gene used was HPRT) from the target CT value. Fold changes in the relative gene expression of the target RNA were determined by calculating the $2^{-\Delta CT}$.

2.2.2 Molecular, Cellular and biochemical methods

2.2.2.1 Western Blot

Electrophoresis: Samples were loaded and electrophoresed through a 5% SDS polyacrylamide stacking gel at 80 V, and then through a 7-15% SDS polyacrylamide resolving gel at 110 V for approximately an hour, or until the dye front ran off the bottom of the gel. The size of the protein determined the percentage of the resolving gel to be used.

Electrotransfer, blocking and antibody incubation: after separation in the polyacrylamide gel, samples were transferred to nitrocellulose membranes using a sandwich system as follows: three pieces of filter paper, membrane, polyacrylamide gel and three more pieces of filter paper, all soaked in Transfer buffer. The ensemble was then placed in a Hoefer TE 70 Semiphor semi-dry transfer unit and run at 200mA for 1.20 min. Subsequently, membranes were blocked with 5% skimmed milk in TBST for 1 hour to avoid non-specific binding of the antibodies. Following three times washing in TBST for 10min, primary antibodies in 5% skimmed milk or BSA (w/v) were added and incubated overnight at 4°C. An additional three washes in TBST was done before adding secondary antibody in 5% skimmed milk and incubation for 1 hour. Membranes were washed in TBST three times for 10min each before detection of bands based on manufacturer's instructions for the secondary antibodies used. Immunoreactive bands were detected through Odyssey infrared imaging system from Licor Biosciences, or by means of E.C.L chemiluminescence reaction in dark room. For proteins with low expression like HIF-1α, anti-mouse or anti-rabbit secondary antibodies containing enzyme horseradish peroxidase (HRP) were used. The chemiluminescent HRP substrate WesternBright™ E.C.L was added to membranes with this type of secondary antibody after the final three washing steps with TBST. The buffer was then drained, and membranes covered with 5mL of working solution mix with equal volumes of WesternBright™ ECL (luminol/enhancer solution) and WesternBright™ Peroxide (stabilized peroxide solution) for 2 min. Membranes were placed between two pieces of plastic sheets in a metal cassette and two to three pieces of ultraradiography film were placed on top for the time required until signals could be determined (range was from 5min to 1.5 hours). Films were then reveal by imbruing the films in X-Ray Developer, followed by X-Ray fixer and finally washed with water at room temperature.

2.2.2.2 ELISA

Cell supernatants were collected following specific treatments and stored at -20°C until ELISA analysis. NUNC "Maxisorb" 96-well plates were coated with the corresponding capture antibodies and incubated overnight at room temperature (See table of concentrations and

diluents below). Plates were then washed three times with wash buffer (PBS with 0.05% Twee-20) and dried. The washing procedure was done in the same way after every step of the ELISA analysis. Plates were blocked for 1h in PBS containing 1% (w/v) BSA. Standard concentrations of analyte were added in duplicates and experimental samples assayed in triplicates. Standards were diluted in reagent diluent with concentrations ranging between 0 and 2000pg/ml depending on the protein to be evaluated. Plates were incubated for at least two hours (at room temperature) or overnight (4°C). Biotinylated detection antibody was added to each well and incubated at room temperature for 2 hours. Streptavidin-HRP conjugate was added and incubated for 20min in the dark. Next, 100µL of liquid substrate TMB ultrasensitive substrate (2.08 mM) solution was added and incubated in the dark for no longer than 20mins. The reaction was stopped with 50 µl per well of 1 NH_2SO_4, and optical depth (OD) was determined by means of an ELx800TM microplate reader and GEN5 Data Analysis Software. The OD was defined for each well at 450nm with correction of 590nm. Standards were used to construct a standard curve with concentrations of samples extrapolated from their OD readings.

Cytokine/Chemokine	**Capture antibody**	**Blocking solution**	**Detection antibody**	**Streptavidin-HRP**
CXCL2/MIP-2 ELISA	1:120 in PBS	Reagent diluent (1% BSA in PBS)	1:60 in Reagent diluent	1:40 in Reagent diluent
IL10 ELISA	1:120 in PBS	Reagent diluent (1% BSA in PBS)	1:60 in Reagent diluent	1:40 in Reagent diluent
IL6 ELISA	1:120 in PBS	Reagent diluent (1% BSA in PBS)	1:60 in Reagent diluent	1:40 in Reagent diluent
KC /CXCL1 ELISA	1:120 in PBS	Reagent diluent (1% BSA in PBS)	1:60 in Reagent diluent	1:40 in Reagent diluent
TNFα ELISA	1:120 in PBS	Reagent diluent (1% BSA in PBS)	1:60 in Reagent diluent	1:40 in Reagent diluent

2.2.2.3 Lactate dehydrogenase (LDH) release

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released upon cell lysis. A non-radioactive colorimetric assay was used to measure LDH release (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega) in supernatants of cells treated with the indicated compounds and ligands for the time points specified in each experiment. The assay consists of a 30-minute coupled enzymatic reaction which produces a red formazan from a tetrazolium salt (INT) conversion. The number of cells lysed is proportional to the colour produced which is determined by means of optical density (OD). The supernatant of at least 3 wells of non-treated cells were used as control for spontaneous LDH release. To establish the maximum release of LDH, triplicate control wells with the same number of cells used per experiment were treated with 20µl of 10X lysis solution (total volume per well was 200µl, final concentration of lysis solution was 1X, diluted in the media) added forty-five minutes prior to the end point of the experimental times. The same volume of warm media (20µl) was added to the experimental wells and controls to compensate for volume differences. Following supernatant collection at the specific time points, 40µl of the supernatants were transferred to a flat-bottom 96-well enzymatic assay plate. Reconstituted Substrate Mix (50µl) was added to each well. Plates were covered and incubated at room temperature in the dark for no longer than 30 minutes. The same volume of Stop Solution was used to end the colorimetric reaction, and absorbance was read at 490nm with correction of 680n in a ELx800TM microplate reader and GEN5 Data Analysis Software. Results were present as percentage of cytotoxicity: (LDH activity (treated cells) – Spontaneous LDH release activity)/(Maximum LDH activity – Spontaneous LDH activity))*100.

2.3 Statistical Analysis

Statistical analysis was done using Prism 5 GraphPad software. Two-tailed Student's t-test was applied where appropriated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

CHAPTER THREE
EXPLORING THE ROLE OF PELLINO 2 AND PELLINO 3 IN REGULATION OF NECROPTOSIS

3.1 Introduction

Regulated cell death is an essential mechanism in the development of organisms. It is also responsible for keeping balance in response to extracellular or intracellular damage threatening homeostasis of a given biological system. The first form of regulated cell death was defined in the early 70s with the description of apoptosis, but more recently, many other types of cell death have been characterized (Galluzzi *et al.*, 2014). Initially, it was believed apoptosis represented the “organized” and highly controlled type of cell death, and necrosis was a more random and uncontrolled process. Nevertheless, in more recent years a regulated form of necrosis has been identified and termed as necroptosis. Being such a definitive fate, all types of cell death have been proven to be tightly regulated. There are several signals of activation or conditions within the cell that are required to initiate cell death. Many proteins are involved in the signalling pathways as facilitators or effector molecules with a family of kinases known as receptor interacting proteins (RIPs) being especially critical in regulating cell death. Given the crucial role of RIP kinases in the finality of a process like cell death, it is hardly surprising that they are subject to tight regulation as well. The activity of RIP kinases is especially dependent on post-translational modifications like ubiquitination. Multiple E3 ubiquitin ligases, proteins mediating the covalent attachment of ubiquitin to target proteins, have been associated with RIP1 post-translational regulation in the context of apoptosis (Humphries *et al.*, 2014). Such E3 ligases include members of the Pellino E3 ligase family. Considering the close interplay between apoptosis and necroptosis, as it will be described in more detail below, the question on how Pellino proteins can regulate other types of cell death was contemplated. Consequently, in this chapter, the role of members of the Pellino E3 ligase family are explored in the context of necroptosis.

3.1.1 Programme cell death

It is generally accepted cell death is a decisive process when loss of cellular function is irreversible. The criteria to define such a definitive mechanism have been widely discussed. For many years, sudden and "chaotic" loss of plasma membrane integrity or “organized” fragmentation and packing of cellular content were the binary forms of cell death. They were for a long time named necrosis and apoptosis or autophagy, respectively. However, many years of research has expanded the knowledge on molecular characteristics allowing for a better

understanding of the mechanisms and subtypes of cell death. Accordingly with the Nomenclature Committee on Cell Death (NCCD) (2015), the main subdivisions for cell death are "accidental" and "regulated" (Galluzzi *et al.*, 2014). The first one, it is a consequence of physical, chemical or mechanical damage, and it cannot be prevented or stopped. Cells dying in this way release damage-associated molecular patterns (DAMPs) which can be recognized by cells of the immune system and trigger cytotoxic responses. The regulated (including programmed) types of cell death have well defined molecular machinery. They are triggered due to unsuccessful efforts to restore cellular homeostasis or as part of developmental evolution of an organism. Alterations in the signalling pathways that control regulated cell death have been associated with several diseases including autoimmunity, infectious diseases and cancer. Some of the regulated types of cell death that have being described include apoptosis, necroptosis (a regulated subtype of necrosis), autophagy, pyroptosis, ferroptosis, among others (**Table 3.1**) (Galluzzi *et al.*, 2014; Kolb *et al.*, 2017). The molecular pathways of some of them are closely related as is the case for apoptosis and necroptosis, the two types of cell death that will be discussed in this section.

3.1.1.1 Apoptosis

Apoptosis was the first type of cell death officially described in early 1970s. It means "falling off" in ancient Greek, and it was characterized by cytoplasmic decline, chromatin condensation, nuclear fragmentation, but most notoriously by the formation of discrete bodies enclosing cytoplasmic content (Elmore, 2007; Galluzzi *et al.*, 2014). It was this last characteristic that defined apoptosis as a "controlled" type of cellular suicide that restricts the spilling of DAMPs and triggering of unwanted inflammatory responses. Apoptosis is not only activated because of damaged cells, but it is also essential to maintain homeostasis in cellular populations and as a developmental mechanism of tissue rearrangement. Two main ways of activation are described for apoptosis, an intrinsic pathway and an extrinsic pathway which are promoted by intracellular and extracellular signals respectively. Intrinsic apoptosis is initiated by cytochrome c and other molecular mediators released from the outer mitochondrial membrane (Tait and Green, 2010). It depends on a loss in balance between proapoptotic and antiapoptotic signals from the BCL2 family of proteins, important in regulation of cell fate (Siddiqui, Ahad and Ahsan, 2015). Intracellular stress signals like DNA damage, accumulation of damaged proteins, hypoxia among others, trigger the permeabilization of mitochondrial membrane and tilts the balance towards proapoptotic signals. Once released, cytochrome c activates downstream signalling through the adaptor APAF-1 and caspase-9 which ultimately turns on effectors caspases like caspase-3 and caspase-7 (**Figure 3.1A**) (Shiozaki, Chai and Shi, 2002; Boatright *et al.*, 2003).

Type of cell death	**Triggers**	**Mediator and effector molecules**	**Final cellular events**
Apoptosis	Intrinsic: developmental signals or stress-induced stimuli. Extrinsic: ligands to extracellular death receptors (DRs) such as TNF, TRAIL, CD95L or FasL.	Proapoptotic and antiapoptotic members of the BCL2 family, initiator caspases (caspase-2, -8, and -9) and executioner caspases (caspase-3, -6, and -7).	DNA fragmentation, chromatin condensation, cell shrinkage and formation of "apoptotic bodies" containing intracellular components.
Autophagy (Autosis)	Response to stress, such as environmental damage, pathogen exposure, metabolic deprivation or chemical fluctuations.	Inhibited by the mTOR complex, class III phosphoinositide 3-kinase (PI3K) complex (Beclin1, VPS34 (the class III PI3K), and VPS15). Effectors: ATG5–12–16L and LC3.	Autophagosomes formation, plasma membrane rupture, disruption of the endoplasmic reticulum and focal swelling up of perinuclear space.
Ferroptosis	Accumulation of toxic radicals. Erastin and RSL3.	Iron-dependant oxidative stress in the cell with increase of reactive oxygen species (ROS) and lipid peroxide species.	Condensation, rupture and shrinkage of the mitochondria. Potentially membrane rupture (yet to be confirmed, described as released of DAMPs).
Necroptosis	RHIM-containing proteins like TLR adapter TRIF and the DNA sensor DAI. Ligands to extracellular death receptors (DRs) such as TNF, TRAIL, CD95L or FasL.	RIP1, RIP3 and MLKL.	Cells swell, and cellular membrane rupture releasing intracellular contents. Mitochondrial swelling and non-condensed chromatin.
Pyroptosis	TLRs and NLRs.	Inflammasomes and inflammatory caspases (like caspase-1), Gasdermin D (GSDMD).	Plasma membrane pore formation and cellular swelling. DNA fragmentation, chromatin condensation.

Table 3.1 Types of programmed cell death and their corresponding triggers, molecular mediators and effectors, and final cellular events. *DAMPs, damage-associated molecular patters; NLRs, nucleotide-binding oligomerization domain-like receptors; TLR, Toll-like receptors* (Adapted from Galluzzi *et al.*, 2014; Kolb *et al.*, 2017).

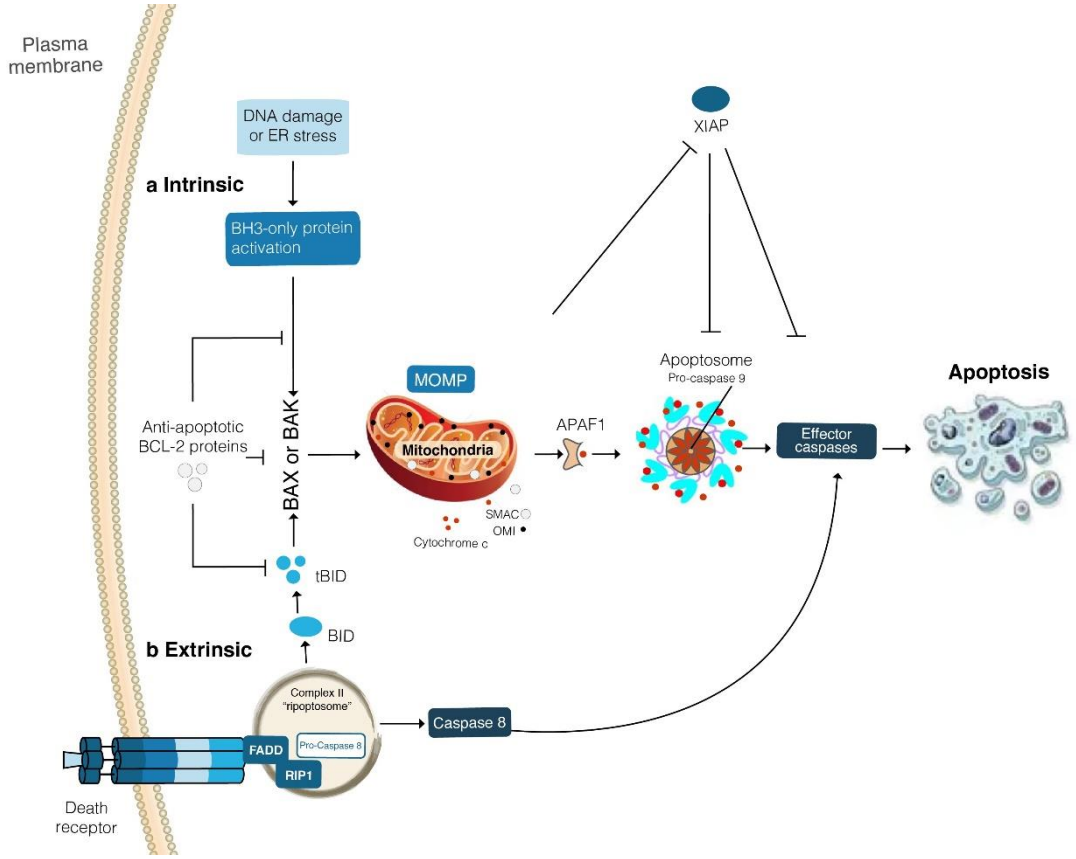

Figure 3.1 Intrinsic and extrinsic pathways of apoptosis.

Two main types of apoptosis pathways have been described: **(A)** intrinsic apoptosis where intracellular signals like DNA damage or endoplasmic reticulum (ER) stress trigger a signalling cascade dependent on permeabilization of the mitochondrial outer membrane and release of cytochrome c and other factors like SMAC and OMI (inhibitors to X-linked inhibitor of apoptosis protein - XIAP). A strict balance between pro-apoptotic and anti-apoptotic members of the B cell lymphoma 2 (BCL-2) family determines mitochondrial permeabilization. In the cytosol, cytochrome c interacts with apoptotic protease-activating factor 1 (APAF1) initiating the formation of an apoptosome, and a subsequent cascade of activated caspases that ultimately leads to cell demise. **(B)** extrinsic apoptosis activated by extracellular signals mainly mediated by stimulation of death receptors. Activation of the later recruits FAS-associated death domain protein (FADD), RIP1 and procaspase 8. This complex, also known as ripoptosome, mediates caspase 8 activation and the cascade of caspase signalling (Adapted from Tait & Green, 2010).

External signals can also stimulate apoptosis by means of death receptors in the extrinsic pathway (**Figure 3.1B**). Tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), and CD95 (Fas/Apo1) are some of the best described activators of death receptors (Wilson, Dixit and Ashkenazi, 2009; Walczak, 2011; Fulda, 2015; Kolb *et al.*, 2017). Engagement of death receptors trigger aggregation of protein complexes at their intracellular domain inducing the formation of a multiprotein complex known as death-inducing signalling complex (DISC) or ripoptosome. Binding of TNF to TNFR1 recruits a series of proteins to its intracellular domain, including TNFR1-associated death domain protein (TRADD) and RIP1. Subsequently, E3 ubiquitin ligases such as TRAF2 and cIAPs1/2 interact with RIP1 mediating its ubiquitination and downstream activation of NF-κB as described previously (See Section 1.1.1.2) (Wilson, Dixit and Ashkenazi, 2009). However, deubiquitination of RIP1 mediated by enzymes CYLD or A20, or absence of cIAPs, blocks RIP1-dependant activation of NF-κB (Declercq, Vanden Berghe and Vandenabeele, 2009; Wilson, Dixit and Ashkenazi, 2009; Humphries *et al.*, 2014). Deubiquitinated RIP1 can then bind to Fas-associated death domain (FADD) and procaspase 8 to form the ripoptosome (Micheau and Tschopp, 2003; Wang, Du and Wang, 2008). In the ripoptosome procaspase 8 is processed to caspase 8 initiating a caspase cascade that leads to cell demise by apoptosis (**Figure 3.1B**) (Wilson, Dixit and Ashkenazi, 2009). There are multiple levels of regulation for the apoptotic pathway. One of them is the absence of cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2), which normally mediate TNFα dependant inflammation by ubiquitinating RIP1 (For detailed explanation on cIAP1/2 mediated TNF-signalling see section 1.1.1.2 of this thesis) (Wang, Du and Wang, 2008; Haas *et al.*, 2009; Feoktistova *et al.*, 2011; Tenev *et al.*, 2011; Huang *et al.*, 2015; Vasudevan and Ryoo, 2015). Another one is the targeting of RIP1 by the Pellino 3 E3 ubiquitin ligase that blocks ripoptosome formation induced by TNF signalling and consequently activation of apoptosis (Yang, Wang, Tang, *et al.*, 2013). Even though the extrinsic and intrinsic pathways initiate apoptosis in different ways, the final executor molecules are the same caspases (**Figure 3.1**). Caspases are the core intermediary proteins of cell death by apoptosis. They are not only mediators in the signalling cascades in apoptosis, they are also their executioners. Caspases are a family of cysteine proteases usually subdivided into initiator (caspase-2, -8, and -9), executioner (caspase-3, -6, and -7) and inflammatory (human caspase-1, -4, and -5) caspases (Elmore, 2007; Shalini *et al.*, 2015; Kolb *et al.*, 2017). Initiator caspases are activated by dimerization and autocatalytic cleavage while executioner caspases gain their proteolytic activity by cleavage and conformational changes (Boatright *et al.*, 2003; Kolb *et al.*, 2017). Once active, executioner caspases cleave over 600 cellular substrates which coordinate dismantling of crucial cellular organelles and structures (Taylor, Cullen and Martin, 2008; Creagh, 2014). Apoptosis has been described as a “silent” type of cellular death, because

one of its main characteristics is the packing of potentially immunogenic cellular content in plasma membrane bodies to avoid activation of inflammatory signals (Taylor, Cullen and Martin, 2008; Kolb *et al.*, 2017). Interestingly, other types of regulated cell death have the opposite function triggering inflammatory responses, with necroptosis being of major current interest.

3.1.1.2 Necroptosis

Contrary to apoptosis, necroptosis has been mainly described as a pro-inflammatory type of cell death. It was described 10 years ago by Hitomi and co-workers as a new form of cell death resembling necrosis, the classical type of accidental cell death, and therefore it was named accordingly (Galluzzi and Kroemer, 2008; Hitomi *et al.*, 2008). Necroptosis is a lytic form of cell death where cells swell, the plasma membrane is disrupted, and intracellular content is spilled releasing DAMPs, just as in necrosis (Galluzzi and Kroemer, 2008). However, a genome-wide siRNA screen for genes regulating the response to death receptors for FasL and TNFα proved it to be a tightly regulated process. Up to 432 genes were recognized in the screen as regulators of necroptosis (Hitomi *et al.*, 2008). The researchers identified an extensive signalling network including genes related to RIP1 kinase activity, death receptor activation, and innate immunity regulation. Further studies have expanded the knowledge on other types of regulated necrosis and described their triggers and specific molecular mechanisms (Vanden Berghe *et al.*, 2014). Proteins containing RHIM domains (like RIP1, RIP3, TLR adapter TRIF and DNA sensor DAI) have been identified as important triggers of necroptosis (Upton, Kaiser and Mocarski, 2012; Kaiser *et al.*, 2013). Activation of the RHIM-domain proteins is initiated by several pathways including agonists for TNFR family of death receptors (TNFR, FASL/ APO-1L, TRAIL, TWEAK), TLRs (TLR3 or TLT4), interferons (IFNs), virus-mediated activation of DNA-dependent activator of IFN-regulatory factors (DAI), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) as well as genotoxic stress and polyclonal stimulation of T cell receptor (TCR) (**Figure 3.2**) (Upton, Kaiser and Mocarski, 2012; Kaiser *et al.*, 2013; Thapa *et al.*, 2013; Dillon *et al.*, 2014; Vanden Berghe *et al.*, 2014). However, the best-known signalling mechanism is the one mediated by death receptor TNFR1.

Signalling triggered by TNFR1 mediates several different responses in the cell depending on the molecular context or the type of cell. Inflammation mediated by TNFα has been broadly described as depending on RIP1 ubiquitination facilitated by E3 ligases like cIAP1/2, TNF receptor-associated factor 2 (TRAF2) or TRAF5 (For detailed explanation on TNFα and TNFR1 signalling see section 1.1.1.2 of this thesis) (Wang, Du and Wang, 2008; Haas *et al.*, 2009; Gyrd-Hansen and Meier, 2010; Feoktistova *et al.*, 2011; Tenev *et al.*, 2011; Huang *et al.*, 2015).

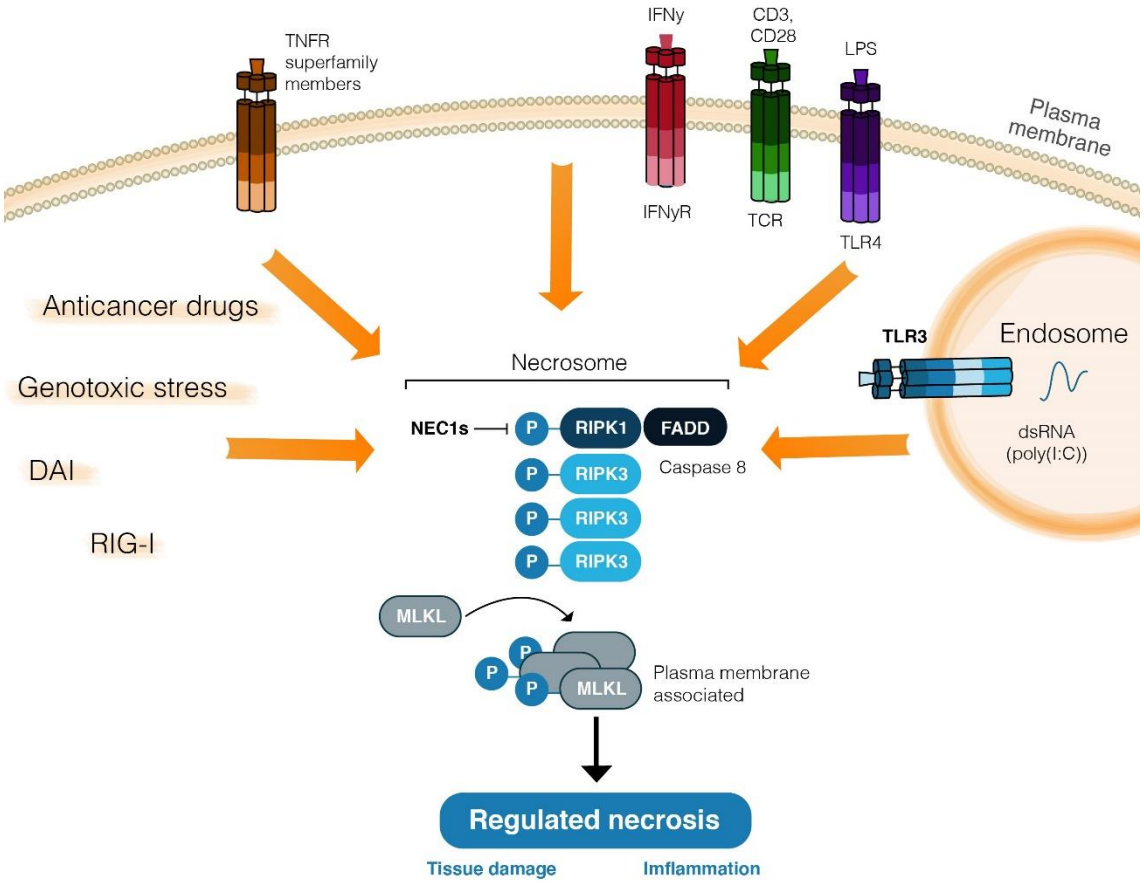

Figure 3.2 Triggers of necroptosis.

Several triggers of necroptosis have been described, including many members of the TNFR family (like TNFR, FASL/APO-1L, TRAIL and TWEAK), TLR3/4, (RIG-I)-like receptors (RLRs), IFN-like receptors, or even genotoxic stress, polyclonal stimulation of TCR (T cell receptor), virus-mediated activation of DNA-dependent activator of IFN-regulatory factors (DAI) or anticancer drugs. Triggering of any of these signalling pathways when caspases are inhibited, allows for RIP3 activation and formation of a necrosome where MLKL is recruited, phosphorylated and mediates the lysis of the plasma membrane (Adapted from Vanden Berghe et al. 2014).

RIP1, in its unubiquitinated form, binds the ripoptosome and triggers the caspase-mediated apoptotic pathway as described previously. Furthermore, when RIP1 cannot be ubiquitinated and the caspases are absent or inactive, RIP1 can interact with RIP3 through their RHIM domains creating an amyloid structure also known as the necrosome (Cho *et al.*, 2009; Li *et al.*, 2012; Vanden Berghe *et al.*, 2014; Wu *et al.*, 2014; Almagro and Vucic, 2015). It was later described that RIP1-RIP3 interaction was required to facilitate RIP3 homo-interaction and auto-phosphorylation (Wu *et al.*, 2014). An extensive amount of evidence came to light supporting the essential role of RIP3 and its kinase activity in necroptosis (Cho *et al.*, 2009; He *et al.*, 2009; Zhang *et al.*, 2009; Newton *et al.*, 2014). Necrosome formation and RIP1-RIP3 interaction within it, allows the recruitment and phosphorylation of mixed lineage kinase domain-like protein (MLKL) (Sun *et al.*, 2012; Rodriguez *et al.*, 2016). Further research showed MLKL is the central effector molecule of necroptotic cell death since MLKL-deficient mice are resistant to this type of regulated cell death (Murphy *et al.*, 2013; Wu *et al.*, 2013). Sun and co-workers (2012), using a small molecule known as necrosulfonamide, that inhibits necroptosis downstream of RIP3, described some of the main characteristics of MLKL and its interaction with RIP3. They showed it is a pseudokinase which binds to RIP3 through its C-terminal kinase-like domain, and the binding depends on phosphorylation of RIP3 in S227 (Sun *et al.*, 2012). Once phosphorylated, MLKL undergoes homo-oligomerization and translocates to the plasma membrane (Sun *et al.*, 2012; Dondelinger *et al.*, 2014). In the membrane, MLKL alters cellular ion homeostasis by binding to phosphatidylinositol phosphates (PIPs) and triggers osmotic cell membrane rupture (Dondelinger *et al.*, 2014; Wang *et al.*, 2014). All necroptotic stimuli induce formation of the necrosome and promote phosphorylation of RIP3 and MLKL with the subsequent rupture of the membrane. However, some of them are independent of TNFR signalling as is the case with TLR3/4 and DAI induced-necroptosis (Upton, Kaiser and Mocarski, 2010, 2012; He *et al.*, 2011; Kaiser *et al.*, 2013). In the case of TLR3/4-induced necroptosis, RIP3 interacts with the RHIM domain in the adapter protein TRIF as a platform for necrosome formation (He *et al.*, 2011; Kaiser *et al.*, 2013). In a similar way, the DNA-sensing receptor DAI also binds to RIP1 and RIP3 through the RHIM domain to activate NF-κB signalling but its necroptotic activity relies only in its binding to RIP3 (Kaiser, Upton and Mocarski, 2008; Upton, Kaiser and Mocarski, 2010, 2012).

Considering necroptosis spills DAMPs as a consequence of membrane rupture, it has been typically described as a pro-inflammatory type of cell death. In that sense it has been suggested that necroptosis may play roles in the pathogenesis of inflammatory diseases like colitis, skin inflammation, retinal degeneration, acute pancreatitis and in transplant rejections (Kaczmarek, Vandenabeele and Krysko, 2013; Pasparakis and Vandenabeele, 2015). However, it

has also been suggested that necroptosis can regulate inflammation by clearing cells and removing pathogens and so limiting their replicative environments (Kaczmarek, Vandenabeele and Krysko, 2013; Kearney *et al.*, 2015). Certainly, necroptosis is an important regulatory mechanism of innate immunity and inflammation. In that sense, a complete understanding of its molecular signalling and how it is regulated becomes necessary. Given RIP Kinases play such a vital role in necroptosis and other types of cell death, their biology will be discussed next.

3.1.2 Protein kinases of the receptor interacting protein (RIP) family

The receptor-interacting protein (RIP) kinase family of proteins have an essential role in activation of immune responses and regulation of cell death (Declercq, Vanden Berghe and Vandenabeele, 2009; Zhang, Lin and Han, 2010; Humphries *et al.*, 2014). They mediate important signals of intracellular and extracellular stress. A homologous N-terminal kinase domain is the common feature of the seven members of this family which contain other specific functional domains (**Figure 3.3**) (Zhang, Lin and Han, 2010; Humphries, 2012). Those specific domains, located at the C-terminal portion of the proteins in most of the family members, seem to underpin the functional diversity of the RIP family. From the seven members, RIP1 to 3 are the best known in terms of functional relevance and molecular signalling, while research on RIP4 to 7 is just in its infancy. RIP1 was the first member of the kinase family to be described in 1995 with a death domain (DD) as its main characteristic (Meylan and Tschopp, 2005). This domain facilitates RIP1 interaction to death receptors containing a similar domain, like FASL/ APO-1L and TNFR, hence its vital role in regulated cell death signalling (Stanger BZ *et al.*, 1995; Hsu *et al.*, 1996). RIP1 also contains an intermediate domain that facilitates its interaction with TNF-receptor-associated factor (TRAF)-1, -2 and -3 (Hsu *et al.*, 1996; Meylan and Tschopp, 2005). Additionally, RIP1 shares a C-terminal RIP homotypic interaction motif (RHIM) domain with RIP3, which allows them to bind and initiate necroptosis (see section 3.1.1.2 Necroptosis). Another member of the family, RIP2, bears a CARD domain associated to caspase activation and recruitment (Meylan and Tschopp, 2005). Like RIP1, RIP2 has an intermediate domain which facilitates its interaction with many TRAF members (Zhang, Lin and Han, 2010). RIP4 and RIP5 also contain intermediate domains as well as ankyrin motifs. The last two members, RIP6 and RIP7, have been described to carry ankyrin, leucine-rich repeat and Roc/COR domains. (Meylan and Tschopp, 2005; Zhang, Lin and Han, 2010; Humphries *et al.*, 2014). Even though the structure of all the RIP family members is well known, the functional and physiological relevance of RIP4-7 is still under research. Some of them have been linked to activation of NF-κB (RIP4), induction of apoptosis (RIP5) and recognition of DAMPs or PAMPs (RIP6/7) (Humphries *et al.*, 2014). Considering the interest of this thesis is regulated cell death, our focus will be on RIP1 and RIP3 signalling.

Given RIP1 was the first member of the RIP kinase family described more than 20 years ago, its interaction with cell death receptors and downstream signalling pathways have been extensively studied. Post-translational modifications of RIP1 are essential to determine its role as a pro-inflammatory signal activating mitogen-activated protein kinases (MAPKs) and NF-κB, or as an inducer of regulated cell death (apoptosis or necroptosis). In the pro-survival scenario,

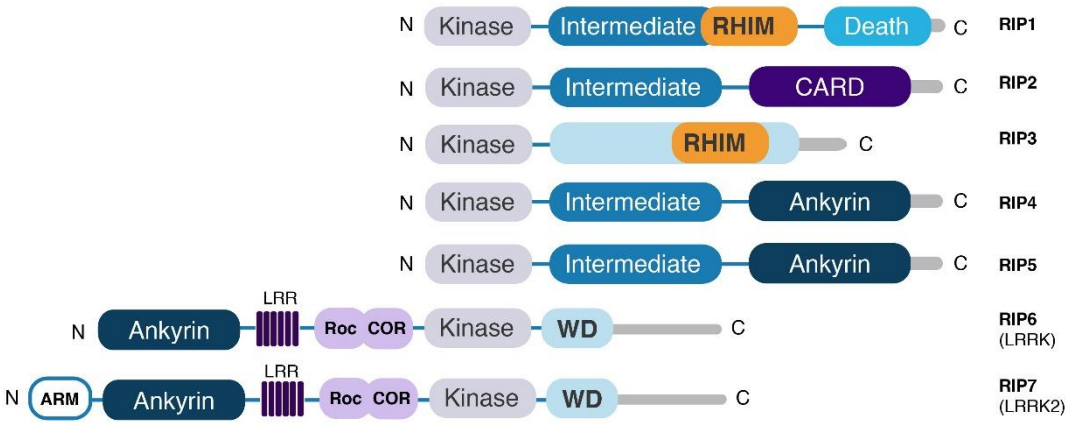

Figure 3.3 Structure of the RIP kinase family.

The domain structures of members of the RIP kinase family are indicated. Roc, Ras of complex proteins; COR, C-terminal of Roc; WD, WD40 repeats; and ARM, Armadillo (From Humphries et al. 2014).

activation of TNF-R1 by TNF recruits TNF-R1-associated death domain protein (TRADD) and RIP1 through its death domain (Hsu *et al.*, 1996). Subsequently, a number of E3 ubiquitin ligases like TRAF2/5 and cIAP1/2 bind to RIP1 and mediate the attachment of a polyubiquitin chain to lysine 63 (K63) (Devin *et al.*, 2000; Lee *et al.*, 2004; Bertrand *et al.*, 2008; Mahoney *et al.*, 2008; Varfolomeev *et al.*, 2008). Furthermore, RIP1 has been suggested to further induce its auto-phosphorylation and ubiquitination in response to TNF signalling (Lee *et al.*, 2004). Once polyubiquitinated, RIP1 binds the ubiquitin-binding domains of proteins that associate with IκB kinase (IKK) and TAK1 (Devin *et al.*, 2000; Chen, 2005). TAK-1 then facilitates activation of IKKs by phosphorylation triggering a signalling cascade that will allow translocation of NF-κB to the nucleus and induction of pro-inflammatory genes (Chen, 2005; Mercurio *et al.*, 2018). Deubiquitination of RIP1 by the deubiquitinases cylindromatosis (CYLD) and A20 or depletion of cIAP1/2 by mediators of depletion like Smac mimetics, switches RIP1 towards cell death mechanisms (Mahoney *et al.*, 2008; Varfolomeev *et al.*, 2008; Declercq, Vanden Berghe and Vandenabeele, 2009; Wilson, Dixit and Ashkenazi, 2009). The deubiquitinated form of RIP1 can then interact with Procaspase 8 and FADD in the ripoptosome or interact with RIP3 in the necrosome (under conditions of caspases depletion) as it was described previously. It is clear that the ubiquitination status of RIP1 plays a critical role in determining the downstream consequences of RIP1 engagement and so much interest has focused on identifying the E3 ubiquitin ligases that catalyse RIP1 ubiquitination. In addition to TRAFs and cIAP E3 ligases, Yang and co-workers described how Pellino 3, another E3 ubiquitin ligase, interacts with RIP1 affecting its capacity to bind the ripoptosome and thereby reducing TNFα-induced apoptosis (Yang, Wang, Tang, *et al.*, 2013).

In addition to TNFR-dependant activation of pro-inflammatory and cell death pathways, RIP1 and RIP3 are also crucial players in TLR signalling pathways, especially those triggered by TLR3 and TLR4. Both of these receptors use an adaptor protein named TRIF to activate NF-κB signalling when stimulated (Yamamoto *et al.*, 2002; Sato *et al.*, 2003; Cusson-Hermance *et al.*, 2005). TRIF recruits RIP1 through their common RHIM domain as part of the signalling activating NF-κB (Meylan *et al.*, 2004; Cusson-Hermance *et al.*, 2005). RIP1 ubiquitination is also necessary in TRIF-mediated activation of NF-κB, and it has been described that Pellino 1, another member of the Pellino E3 ligase family, is responsible for its post-translational modification in the context of TLR3 and TLR4 signalling (Chang, Jin and Sun, 2009). In a similar way to TNFR-dependant signalling, in the absence of caspases, the adaptor TRIF can bind directly to RIP3 through the RHIM domain and, thereby, initiate necroptosis by stimulation of TLR3 and TLR4 (He *et al.*, 2011;

Kaiser *et al.*, 2013). Clearly, the E3 ubiquitin ligase Pellino family has an important role in regulation of RIP kinases, therefore, their interaction will be discussed next.

3.1.3 E3 ubiquitin ligase Pellino family

3.1.3.1 Ubiquitin enzyme system

The regulation of protein degradation is critically important in controlling cell signalling pathways. Multiple proteins are implicated in the complex system that mediates recognition, labelling and subsequent degradation of the proteins to be discarded. This set of proteins and molecular mechanism is known as the ubiquitin proteasome system (UPS) (Komander and Rape, 2012; Inobe and Matouschek, 2014). Proteins to be degraded are labelled with ubiquitin tags or degrons. Ubiquitination is not only a signal to degrade or not degrade a protein, the nature and complexity of the labelling also indicates the order and time of degradation (Deshaies and Joazeiro, 2009). The covalent binding of polypeptide chains of ubiquitin, a 76 amino acids protein, to the ε-amino group of lysine residues in target proteins allows for their proteasome recognition and degradation (Ravid and Hochstrasser, 2008; Komander and Rape, 2012). Diversity of branched or linear polyubiquitin chains and the specific lysine or other residue where it is attached determines whether target proteins are marked for degradation or as signalling-related modification (Ravid and Hochstrasser, 2008; Ernst *et al.*, 2013; Swatek and Komander, 2016). For example, Lys48-linked polyubiquitin chains are usually associated with proteasomal degradation while Lys63-linked chains are described as anchor site for signalling complexes (Swatek and Komander, 2016). A group of enzymes catalyze the sequential process of ubiquitylation: ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3) (**Figure 3.4A**) (Komander and Rape, 2012). Activating enzymes (E1) forms a thioester bond with ubiquitin in an ATP-dependent way, then conjugating (E2) enzymes accept the ubiquitin from E1. Finally, E3 ubiquitin ligases recognizes the target protein and binds the ubiquitin-loaded E2 enzyme, to then catalyze the attachment of polyubiquitin chains (Deshaies and Joazeiro, 2009). Proteins can be ubiquitinated in different residues as it is the example of RIP1 that can be ubiquitinated in seven different residues depending on its interaction with specific E2-E3 ligases (**Figure 3.4B**). E2-E3 complexes are responsible for the diversity in the modification on target proteins (Metzger *et al.*, 2014). While only 2 subtypes of E1 enzymes have been described approximately 40 E2s and between 500 and 1000 human E3s are recognized (Pelzer *et al.*, 2007; Komander and Rape, 2012; Stewart *et al.*, 2016; Swatek and Komander, 2016). E3 ligases are a highly conserved, wide and diverse group of enzymes. In eukaryotes, mainly two types are recognized: HECT or RING domain. Up to 616 different types of RING domain ligases have been described to be expressed in human cells (Deshaies and Joazeiro, 2009; Komander and Rape, 2012). Considering the Pellino E3 ligase family has been linked to RIP kinases and regulated cell death, it will be the focus of this thesis.

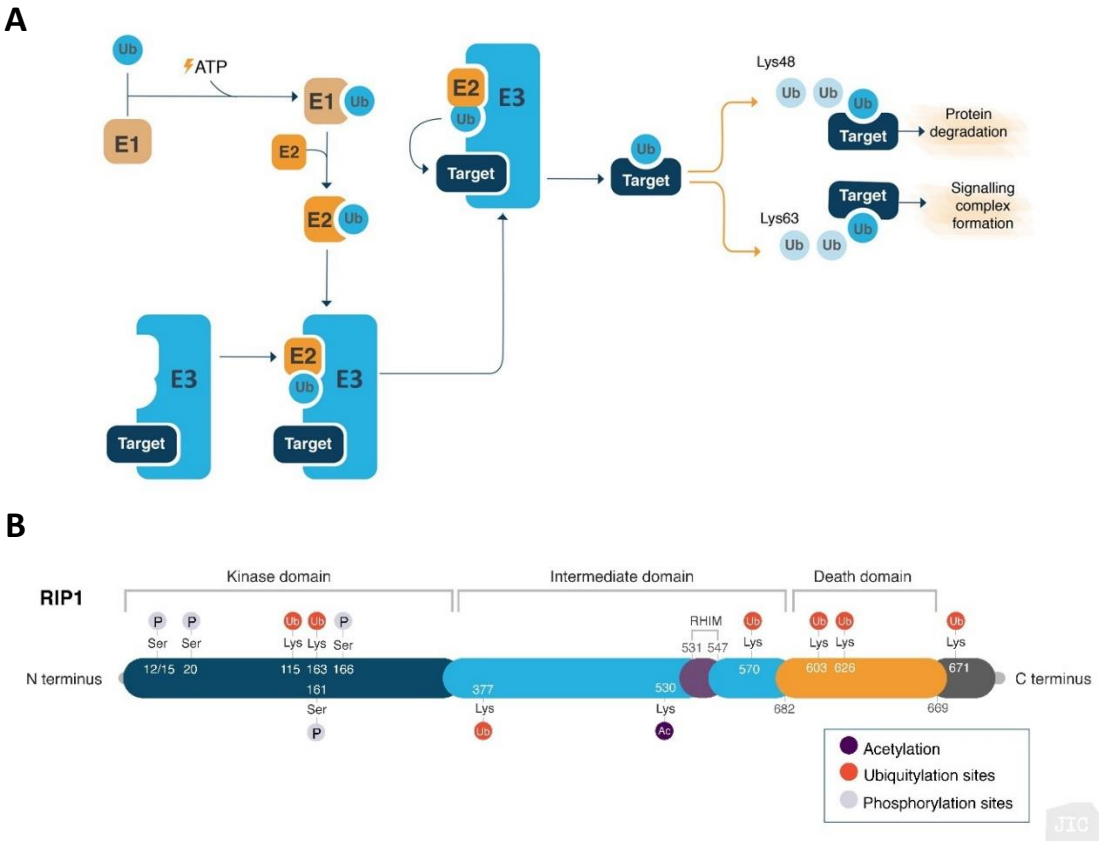

Figure 3.4 The ubiquitylation pathway.

(A) The ubiquitylation pathway mediates the binding of a isopeptide chain made of ubiquitin to a target protein as a mark for proteasome-dependent degradation or as a post-translational modification to regulate its function, structure or location. The polyubiquitin chains are mostly attached to lysine residues in the C-terminal portion of the target protein. The complexity and nature of the polyubiquitin chain and the residue where it is attached determines the biological function of the mark. For example, Lys48-linked ubiquitin chains is a signal for proteasomal degradation while Lys63-linked chains facilitates the formation of signalling complexes. This process is catalyzed by a series of enzymes known as ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). E1 and E2 facilitate the binding and loading of ubiquitin molecules while E3 ligases catalyze the ligation of the same to target proteins (Adapted from Moynagh, PN. 2014). **(B)** Example of the different point of ubiquitination of RIP1. RIP1 can be ubiquitinated in seven residues in its kinase, intermediate and C-terminal death domain. RIP1 can also be post-translationally modified by phosphorylation and acetylation. It has been described that ubiquitin-binding the death domain are essential for signalling associate to the death receptor and the apoptotic pathway, while ubiquitin-binding the intermediate domain mediates NF-κB signalling and necroptosis (Adapted from Ofengeim and Yuan. 2013).

3.1.3.2 Pellino family

Pellino proteins form a family of E3 ubiquitin ligases with a C-terminal RING domain and a N-terminal forkhead-associated (FHA) domain containing wing structures specific to this family (**Figure 3.5**) (Lin *et al.*, 2008; Moynagh, 2014). The RING-domain has been indicated as the portion of the protein responsible for the E3 ubiquitin catalytic activity mediating the Lys11, Lys48 or Lys63 polyubiquitination of target proteins while the FHA-domain recognizes and binds phosphorylated targeted proteins (Moynagh, 2014). Three members have been identified in mammalians: Pellino 1, Pellino 2 and Pellino 3 (L. E. Jensen and Whitehead, 2003; Liselotte E. Jensen and Whitehead, 2003; Strelow, Kollewe and Wesche, 2003; Moynagh, 2014). Interestingly, Pellino proteins were first identified as scaffold proteins in TLR/IL-1R signalling, interacting with interleukin-receptor-associated kinase (IRAK) and MAPKs (L. E. Jensen and Whitehead, 2003; Liselotte E. Jensen and Whitehead, 2003; Strelow, Kollewe and Wesche, 2003; Butler, Hanly and Moynagh, 2005). Later, it was described that they have E3 ubiquitin ligase functions with Pellino-mediated ubiquitination of IRAK1 being demonstrated in vitro (Schauvliege, Janssens and Beyaert, 2006; Butler, Hanly and Moynagh, 2007). All members of this family can interact with several E2 enzymes to mediate Lys11-, Lys48- and Lys63-linked polyubiquitin chains (Ordureau *et al.*, 2008). Activation of Pellino's E3 ligase activity seems to be specific to the signalling pathway being initiated. IRAK proteins have been described to meditated Pellino phosphorylation and activation in the context of IL1 stimulation while TBK1/IKKε-mediated phosphorylation of Pellinos has been associated to TNFR1 and TLRs signalling pathways (Devin *et al.*, 2000; Chen and Goeddel, 2002; Goh *et al.*, 2012).

The Pellino family of E3 ligases has been shown to interact with RIP kinases family in a context-dependent way. Pellino 1 has proven to mediate Lys63 polyubiquitination of RIP1 in a TRIF-dependant way in macrophages activated through TLR3 or TLR4 (Chang, Jin and Sun, 2009). Ubiquitination of RIP1 in Lys63 allows for the recruitment of transforming growth factor b-activated kinase 1 (TAK1) which associates and phosphorylates the IκB kinase (IKK) complex releasing NF-κB (See sections 1.1.1.2 and 1.1.2.2 from general introduction) (Devin *et al.*, 2000; Chen, 2005; Chang, Jin and Sun, 2009; Mercurio *et al.*, 2018). Interestingly, Pellino 3 also interacts with RIP1 but in a completely different context. Pellino 3 deficiency sensitizes cells to TNF-induced apoptosis (Yang, Wang, Tang, *et al.*, 2013). Yang and co-workers (2013) described a Pellino 3 FHA domain-dependant interaction with RIP1 triggered by TNF signalling which restricts RIP1 binding to FADD and pro-caspase 8 and therefore ripoptosome formation. It is to note that Pellino1 and Pellino 3 interact with RIP1 in a domain-specific way to trigger two different responses, with Pellino1 promoting activation of NF-κB and

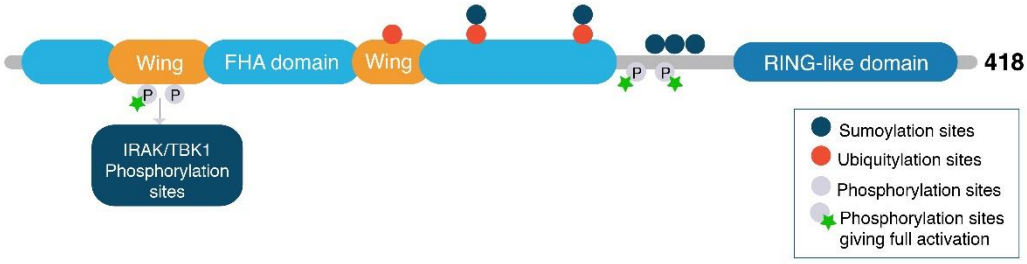

Figure 3.5 Pellino domain structure.

This E3 ligase family contains a core forkhead-associated (FHA) domain with two inserts that form a 'wing' and a RING-like domain. The sites for ubiquitylation (Ub), sumoylation (Su) and phosphorylation (P) are indicated based on their corresponding sites of occurrence in Pellino 1. Sites phosphorylated particularly by interleukin-1 receptor-associated kinase (IRAKs) and TANK-binding kinase 1 (TBK1) are in the wing domain at the N-terminal portion of the protein. The green stars specify phosphorylation sites that fully activate the E3 ubiquitin ligase activity of Pellino 1. Addition of phosphate molecules into those particular phosphorylation sites facilitates a conformational change in the structure of Pellino proteins allowing its interaction with E2 ligases and the target protein, and therefore mediating its functional activation (Adapted from Moynagh, PN. 2014).

Pellino 3 regulating a type of cell death. Furthermore, Pellino 3 has been linked to RIP2 kinase in a model of experimental colitis. In this model, Pellino 3 was showed to mediate the response of the intracellular receptor NOD2 by enabling Lys63 polyubiquitination of RIP2 (Yang, Wang, Humphries, *et al.*, 2013). NOD1 and NOD2 are members of the pattern recognition receptors (PRRs) and recognize bacterial peptidoglycans (Kumar, Kawai and Akira, 2011). NOD2 signalling requires RIP2 ubiquitination by Pellino 3 and X-Linked Inhibitor of Apoptosis Proteins (XIAPs) leading to recruitment of TAK1 and IKK complexes and activation of NF-κB and MAPKs (Humphries *et al.*, 2014). Ubiquitination of RIP2 is essential for NOD2 signalling and its activation of NF-κB (Yang *et al.*, 2007; Hasegawa *et al.*, 2008). Whilst Pellinos play roles in inflammation and in the case of Pellino 3 in apoptosis there are emerging reports for a role for Pellino 1 in necroptosis. Pellino 1 has been recently described to modulate necroptosis signalling by regulating both RIP1 and RIP3 (Wang *et al.*, 2017; Choi *et al.*, 2018). The latter report shows that ubiquitination of RIP1 by Pellino 1 is indispensable for its interaction with RIP3 and the subsequent formation of the necrosome and consequently initiation of necroptosis. Contrastingly, Choi and co-workers recently reported that Pellino 1 is a negative modulator of necroptosis by mediating the degradation of RIP3 (Choi *et al.*, 2018). The basis to these contrasting reports are unknown. The initial studies in this thesis explore the role of Pellino 2 and Pellino 3 in controlling cell death, especially by necroptosis.

3.2 Results

3.2.1 Effect of Pellino 2 and/or Pellino 3 deficiency on TNFα-induced necroptosis in Mouse Embryonic Fibroblast (MEFs)

Considering Pellino 3 has been proven to regulate RIP1 and RIP2, and knowing RIP kinases are essential mediators of cell death, the present Chapter aimed to explore the role of Pellino 3 and another family member Pellino 2 in the regulation of necroptosis (Yang, Wang, Humphries, *et al.*, 2013; Yang, Wang, Tang, *et al.*, 2013; Humphries *et al.*, 2014). As previously described, necroptosis is a type of necrosis tightly regulated and dependent on interaction between RIP1 and RIP3 (Hitomi *et al.*, 2008; Vanden Berghe *et al.*, 2014; Silke, Rickard and Gerlic, 2015). In order to study necroptosis, cells are treated with a combination of two different inhibitors (Z-VAD and BV6) for 1 hour and then stimulated with TNFα for 6 or 24 hours. Z-VAD is a well-known pancaspase inhibitor and BV6 inhibits cellular IAPs by inducing their autoubiquitination and subsequent degradation by the proteasome (Müller-Sienerth *et al.*, 2011; Vasudevan and Ryoo, 2015). The blockade of cIAP–mediated ubiquitination of RIP1 prevents the latter from promoting downstream activation of NF-κB and instead directs it to cell death pathways. However, Z-VAD prevents apoptosis and instead RIP1 interacts with RIP3 to trigger the signalling cascade for necroptosis. Phosphorylation of MLKL, the main effector molecule for necroptosis, as well as decrease in RIP1 expression and upward shift of RIP3 due to phosphorylation are expected as manifestation of this cell death mechanism.

The first study explored the role of Pellino 2 in regulating TNF-induced necroptosis in murine embryonic fibroblasts (MEFs). WT or Pellino 2-deficient MEFs were treated alone or in combination with TNFα, Z-VAD and BV6 for an acute period of 6 hours. The combination of all 3 treatments promoted high levels of P-MLKL consistent with this treatment regime causing necroptosis in MEFs (**Figure 3.6A**). Indeed, low levels of P-MLKL were also apparent when cells were treated only with BV6 and TNFα, indicating some necroptosis even in the absence of suppression of caspase activity. As for the RIP kinases, levels of RIP1 decreased with the different combinations of inhibitors, with the RIP1 immunoreactive band almost disappearing with Z-VAD, BV6 and TNFα. Meanwhile, RIP3 expression was evident for all the combinations of treatments. However, when the cells were driven toward necroptosis with all 3 agents, an upward shift was evident for RIP3, possibly due to changes in size derived from its phosphorylation and subsequent formation of the necrosome complex. Similar patterns of expression of P-MLKL, RIP1 and RIP3 were also observed in response to these treatments in *Peli2*$^{-/-}$ MEFs. However, the

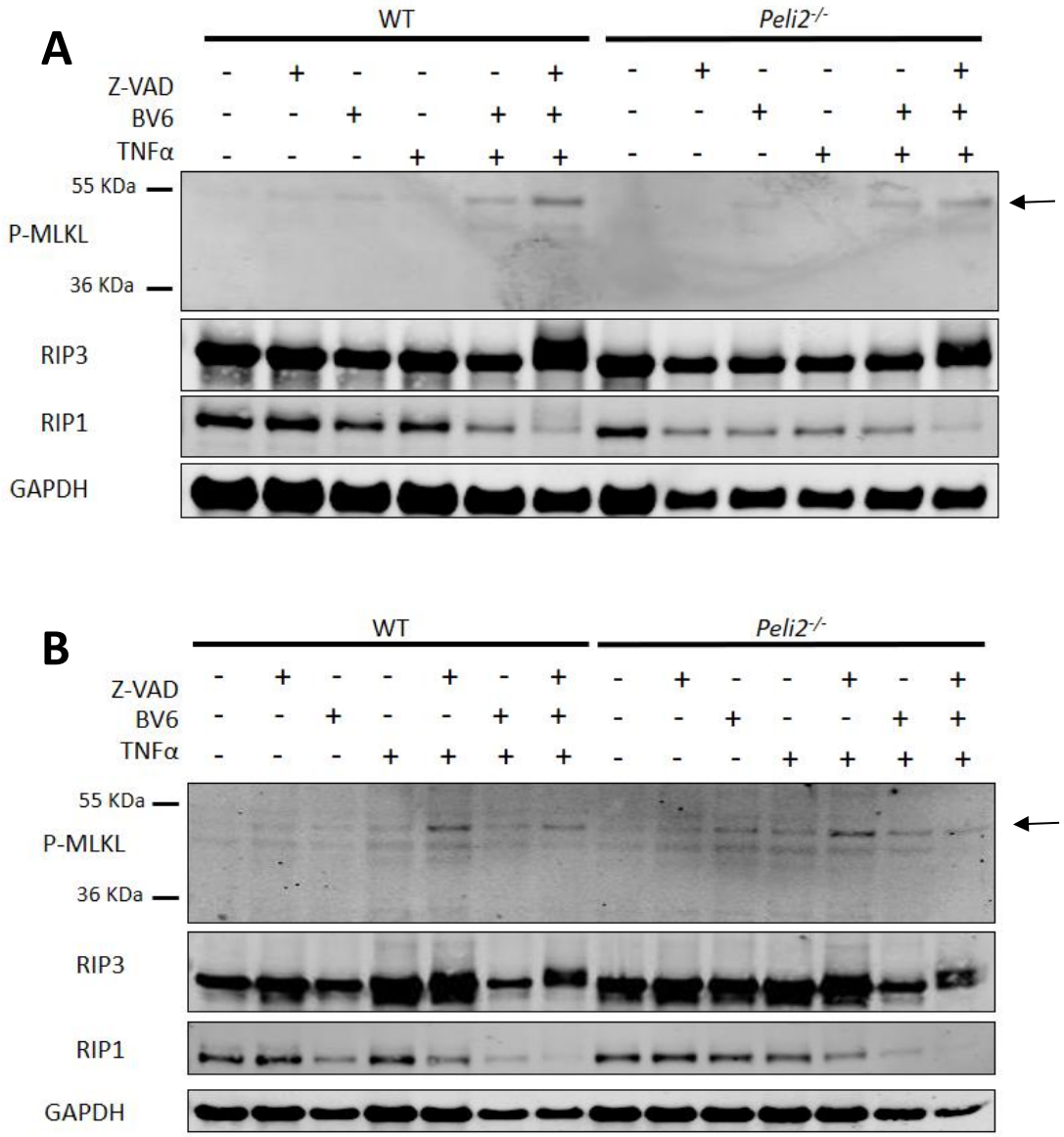

Figure 3.6 Pellino 2 deficiency has moderate effect on molecular markers of necroptosis induced with TNFα in murine embryonic fibroblasts (MEFs).

Immunoblot analysis of phosphorylated (p-) MLKL (54 kDa) and total levels of RIP1 (74 kDa) and RIP3 (46-53 kDa) in cell lysates from WT and *Peli2*$^{-/-}$ MEFs pre-treated with 20 µM Z-VAD for an hour and stimulated with 10 µM BV6 and 40 ng/mL TNFα for **(A)** 6 hrs and **(B)** 24 hrs. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

levels of P-MLKL in *Peli2*$^{-/-}$ MEFs in response to all 3 treatments were slightly less than corresponding samples from WT cells suggesting that Pellino 2 may play some modest part in TNF-induced necroptosis (**Figure 3.6A**). Variations in the levels of housekeeping molecules like β-actin and GAPDH were apparent in experiments, specifically at long time points with combined treatment. However, considering cells were being driven towards cell death, these observations were anticipated as proteins were degraded and cellular content lost.

Similar experiments were also performed in which MEFs from WT and *Peli2*$^{-/-}$ MEFs were exposed to the different treatments for a longer period of 24 hours (**Figure 3.6B**). Similar profiles were observed as described above for 6h exposure to the various treatments. However, WT and *Peli2*$^{-/-}$ MEFs displayed the same responsiveness to the treatments suggesting that Pellino 2 is not involved in TNF-induced necroptosis over a more prolonged time frame. In addition to measuring molecular markers for necroptosis, the same model of treatment was used to measure cytotoxicity by assaying cell supernatants for levels of lactate dehydrogenase (LDH) that were released by dying cells. In this case, not only BV6 was used, but other two IAPs inhibitors (LCL161 and Birinapant) were tested to compare the effects of their inhibitory affinities for IAPs in the induction of cell death. Cellular IAPs 1 and 2 (cIAP1/2), and X-linked IAPs (XIAPs), have been described to regulate effector caspases and apoptosis and also TNFα and TLRs-mediated signalling (Vasudevan and Ryoo, 2015). The three IAPs inhibitors used in this research have been reported to have specific affinities for the two types of IAPs (cIAPs and XIAPs). BV6 blocks c-IAPs 1 and 2 but not XIAPs, whilst Birinapant targets c-IAPs and XIAPs but with lower affinity for the latter (Condon *et al.*, 2014; Lawlor *et al.*, 2015). On the other hand, LCL161 displays high affinity for c-IAPs 1 and 2 and XIAP (Lawlor *et al.*, 2015). To compare the cytotoxicity induced by the inhibitors, WT and *Peli2*$^{-/-}$ MEFs were treated with Z-VAD and either LCL161, BV6 or Bririnapant for 1 hour and then stimulated with TNFα for 24 hours (**Figure 3.7**). Treatment of WT cells with LCL161 alone was seen to induce cytotoxicity up to 50%, while the combined treatment of LCL161 with TNFα increased it to 60% and with Z-VAD and TNFα to approximately 65% (**Figure 3.7A**). *Peli2*$^{-/-}$ MEFs showed the same pattern of cytotoxicity as seen in WT when treated with LCL161 or any of the combined treatments with Z-VAD and TNFα. In contrast, cytotoxicity levels for WT MEFs treated with BV6 were low (approximately 20%) but when combined with TNFα or Z-VAD and TNFα considerably increased up to 70% and 80% respectively (**Figure 3.7B**). Pellino 2 deficient cells treated with BV6 or Z-VAD, BV6 and TNFα showed the same percentages of cytotoxicity as WT cells. However, there was a small reduction of up to 10% when the *Peli2*$^{-/-}$ MEFs were treated with BV6 and TNFα compared with WT cells. Finally, Birinapant by itself induced 30% cytotoxicity in WT MEFs (**Figure 3.7C**). Combined with

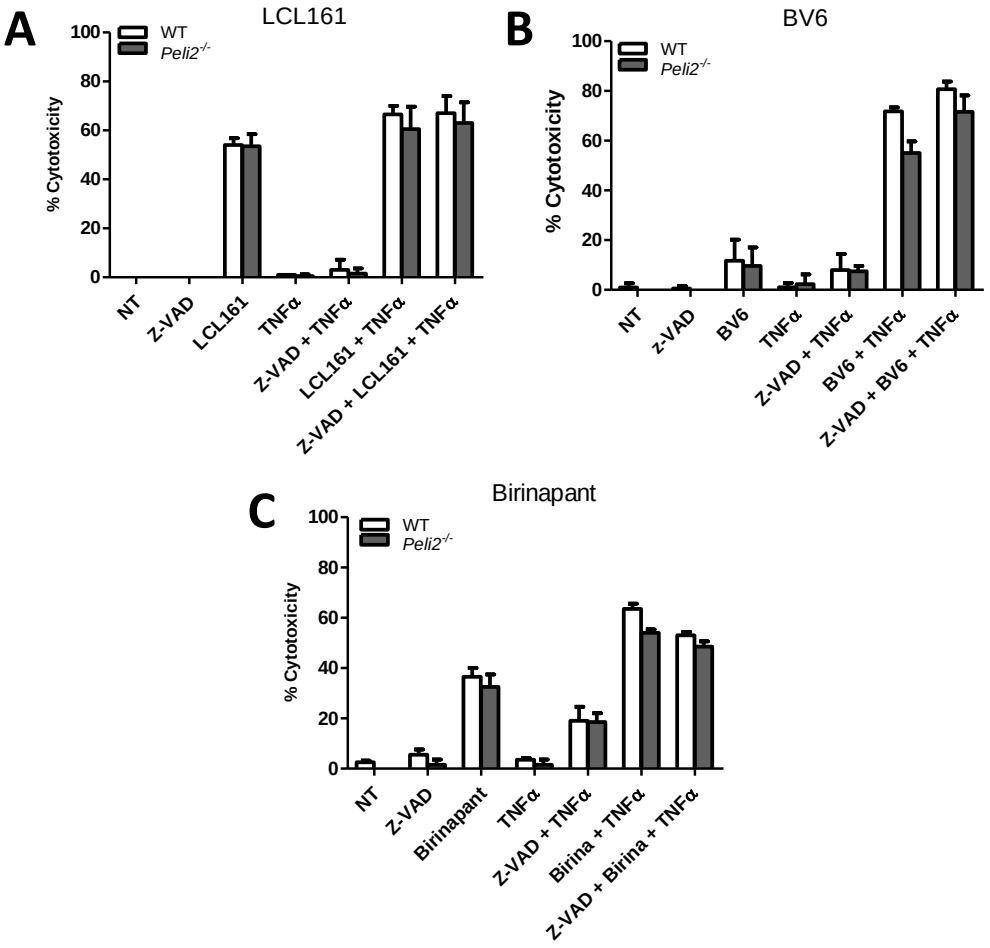

Figure 3.7 Pellino 2 deficiency does not affect TNF-induced necroptosis as measured by release of lactate dehydrogenase in MEFs.

Cytotoxicity analysis by assay of LDH release in supernatant from WT and *Peli2*$^{-/-}$ MEFs pre-treated with 20 µM Z-VAD for an hour and stimulated with 40 ng/mL TNFα and **(A)** 10 µM BV6, **(B)** 10 µM LCL161 or **(C)** 10 µM Birinapant (Birina) for 24 hrs. Data are presented as the mean ± SEM of two independent experiments. Maximum release of LDH was stablish for each biological replicate using lysis buffer provided with the LDL assay Kit. Additional details can be found in Section 2.2.2.3.

TNFα or Z-VAD and TNFα the percentages were 60% and 50% respectively. However, Pellino 2 deficient cells showed the same levels of cytotoxicity as WT cells with a minor decrease when cells were treated with Birinapant and TNFα. All together these results question the role, if any, of Pellino 2 in TNF-induced necroptosis.

The next step was to evaluate the role of another Pellino family member, Pellino 3, in the regulation of TNF-induced necroptosis in MEFs. Thus, WT and *Peli3*$^{-/-}$ MEFs were pre-treated with Z-VAD and BV6 for an hour and then stimulated with TNFα for 6 hours (**Figures 3.8A**). The same molecular markers of necroptosis were tested as described above. Phosphorylation of MLKL was observed when cells were treated with Z-VAD or BV6 and TNFα, and with both inhibitors and TNFα. It was also present with BV6 treatment alone. WT and *Peli3*$^{-/-}$ MEFs showed the same patterns and profiles of P-MLKL immunoreactivity indicating that Pellino 3 is not involved in TNF-induced necroptosis. As before the slower migrating form of RIP3 was also evident for WT cells driven toward necroptosis by combined treatment of all 3 agents but this was also apparent in Pellino 3-deficient MEFs. RIP1, was also depleted in cells that were induced to progress to either apoptosis and necroptosis. However, there was no difference between WT and Pellino 3 knockout MEFs. Similar results were visible at 24 hours treatment (**Figures 3.8B**). Variations in the housekeeping protein, GAPDH, are explained by the strong induction of cell death in such a long period of treatment. These data indicate a lack of involvement of Pellino 3 in regulation of necroptosis, at least in fibroblasts.

Given the highly conserved nature of the primary and predicted secondary structures of the Pellino family members, studies next investigated if the lack of effect of Pellino 2 or Pellino 3 deficiency in TNF-induced necroptosis may be explained by functional redundancy of the 2 family members. To this end double knockout mice were generated by pairing homozygous *Peli2*$^{-/-}$ and *Peli3*$^{-/-}$ for breeding and subsequently genotyping these alleles in each mouse as described in the materials and methods (see sections 2.1.4 and 2.1.7). Additionally, double knockout Pellino 2 and 3 MEFs were assessed to rule out a potential compensation effect between both members of the family (**Figure 3.9**). As for previous experiments, phosphorylation of MLKL was apparent under conditions of apoptosis (TNFα and BV6) and more prominently under conditions of necroptosis (TNFα, BV6 and Z-VAD) (**Figure 3.9**). The upward shift in the RIP3 immunoreactive band and reduced levels of RIP1 again coincided with these changes. However, WT and double knockout MEFs showed the same patterns and levels of P-MLKL, RIP1 and RIP3 again confirming the absence of a role for Pellino 2 or Pellino 3 in TNF-induced necroptosis in MEFs.

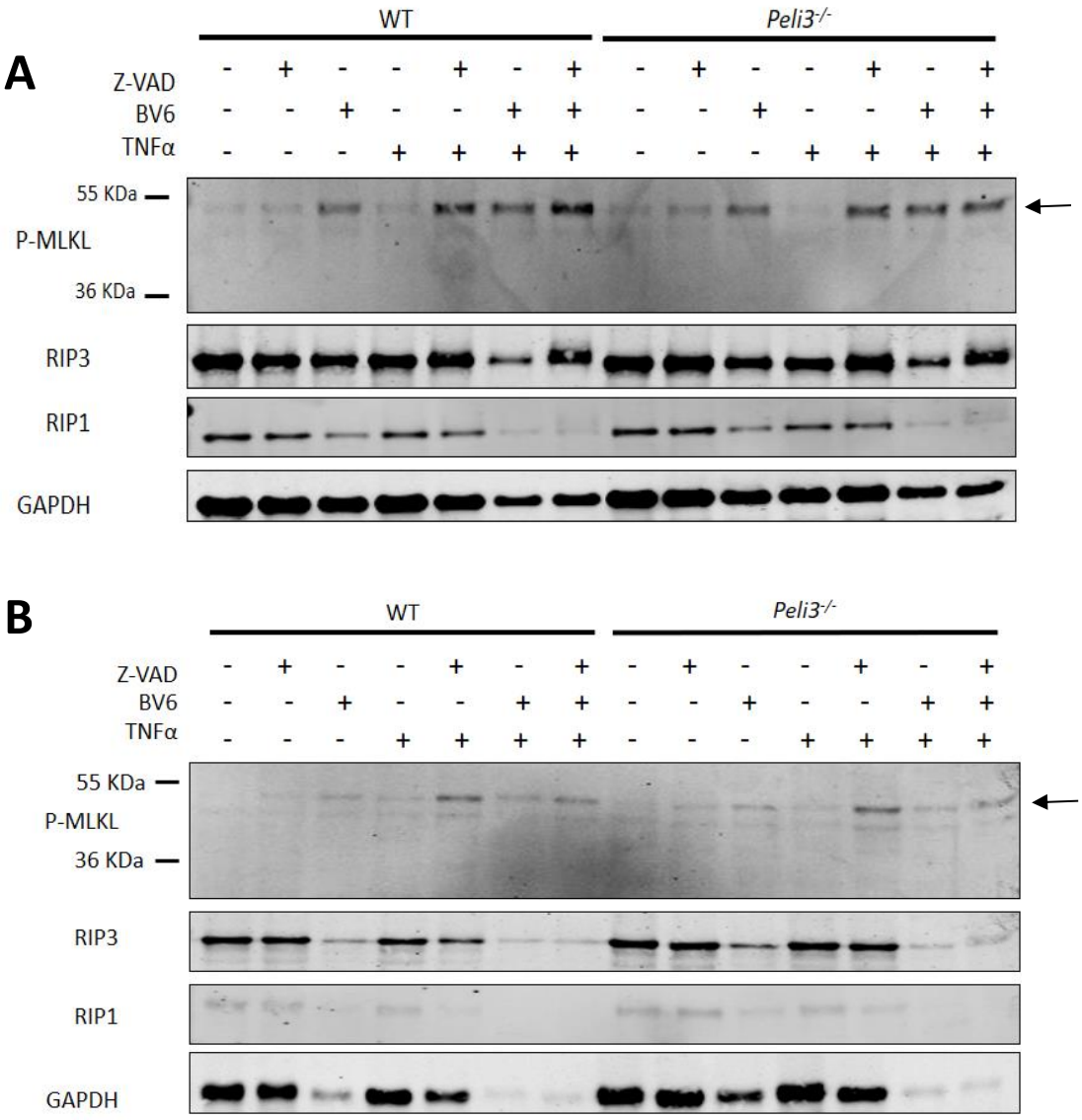

Figure 3.8 Pellino 3 deficiency has no effect on molecular markers of necroptosis induced with TNFα in MEFs.

Immunoblot analysis of phosphorylated (p-) MLKL (54 kDa) and total levels of RIP1 (74 kDa) and RIP3 (46-53 kDa) in cell lysates from WT and *Peli3*$^{-/-}$ MEFs pre-treated with 20 µM Z-VAD for an hour and stimulated with 10 µM BV6 and 40 ng/mL TNFα for **(A)** 6 hrs and **(B)** 24 hrs. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

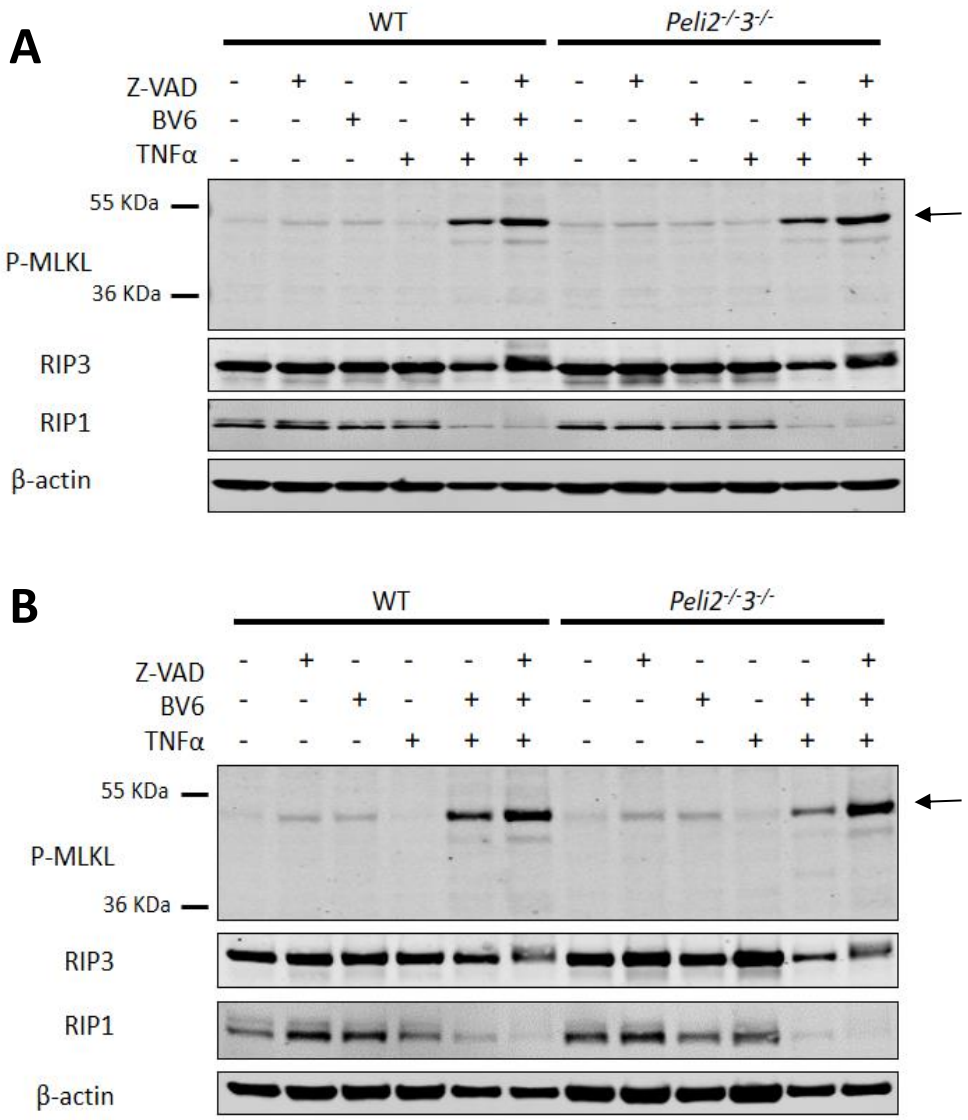

Figure 3.9 Pellino 2 and Pellino 3 deficiency has no effect on molecular markers of necroptosis induced with TNFα in MEFs.

Immunoblot analysis of phosphorylated (p-) MLKL (54 kDa) and total levels of RIP1 (74 kDa) and RIP3 (46-53 kDa) in cell lysates from WT and *Peli2*$^{-/-}$*Peli3*$^{-/-}$ MEFs pre-treated with 20 µM Z-VAD for an hour and stimulated with 10 µM BV6 and 40 ng/mL TNFα for **(A)** 8 hrs and **(B)** 24 hrs. β-actin (42 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

3.2.2 Effect of Pellino 2 and Pellino 3 deficiency in TNFα-induced necroptosis in immune cells

Although Pellino 2 and 3 appear to play negligible roles in regulation of TNF-induced necroptosis in embryonic fibroblasts, we were keen to examine their roles in this pathway in immune cells. Macrophages are essential in the initiation of immune responses, and more likely encounter signals triggering cell death due to their phagocytic function. Therefore, bone marrow-derived macrophages (BMDMs) were treated in the same way as MEFs in previous section. LCL161 was selected as the IAP inhibitor of choice since it is a pan cIAP/xIAP inhibitor and also preliminary experiments showed a lack of response of BMDMs to BV6 (Condon *et al.*, 2014; Lawlor *et al.*, 2015). WT BMDMs treated with only Z-VAD or LCL161 for 6 hours induced low level phosphorylation of MLKL, but this was strongly augmented with triple treatment of Z-VAD, LCL161 and TNFα (**Figure 3.10A**). The RIP3 and RIP1 expression patterns in WT BMDMs was largely similar to MEFs, with the RIP3 band showing upward migration when necroptosis was induced with Z-VAD, LCL161 and TNFα. RIP1 significantly decreased with LCL161 treatment alone, or when treated with LCL161 plus TNFα, or Z-VAD, LCL161 and TNFα. Notably the levels and migration patterns of pMLKL, RIP1 and RIP3 in WT BMDMs were replicated in *Peli2*$^{-/-}$ BMDMs (**Figure 3.10A**) and *Peli3*$^{-/-}$ BMDMs (**Figure 3.10B**). A kinetic study was also performed in which WT, *Peli2*$^{-/-}$ and *Pel3*$^{-/-}$ were subjected to the triple co-treatment of Z-VAD, LCL161 and TNFα and assessed for markers of necroptosis as described above. The same levels of MLKL phosphorylation, time dependant decrease of RIP1 and upward shift of RIP3 were apparent in WT BMDMs, *Peli2*$^{-/-}$ BMDMs and *Pel3*$^{-/-}$ BMDMs (**Figure 3.10C**). These results clearly indicate that neither Pellino 2 or Pellino 3 have a role in the regulation of TNF-induced necroptosis in BMDMs.

LDH release was also used as a measure of cytotoxicity and necroptosis and evaluate the role of Pellino 2 and Pellino 3 in TNF-induced necroptosis in macrophages. These analyses lend themselves to higher throughput analysis than the approaches above that rely on immunoblotting. The cytotoxicity induced by IAPs inhibitors LCL161 and Birinapant when combined with TNFα or Z-VAD and TNFα was evaluated in WT and *Peli2*$^{-/-}$ BMDMs (**Figure 3.11A**), and WT and *Peli3*$^{-/-}$ BMDMs (**Figure 3.11B**). The cells were treated with Z-VAD and either LCL161 (left panels) or Bririnapant (right panels) for 1 hour and then stimulated with TNFα for 24 hours (**Figure 3.11**). Treatment with LCL161 alone, LCL161 plus TNFα or Z-VAD, LCL161 and TNFα increased cytotoxicity to 80% in WT BMDMs with no differences in the percentages of cytotoxicity when compared with *Peli2*$^{-/-}$ BMDMs subjected to the same treatments (**Figure 3.11A, left**). Cytotoxicity in WT and *Peli2*$^{-/-}$ BMDMs induced by Birinapant only or combined with TNFα or Z-VAD and TNFα increased up to 60% but with no difference between WT and *Peli2*$^{-/-}$ BMDMs (**Figure 3.11A, right**). Pellino 3 deficient cells also induced the same percentages of

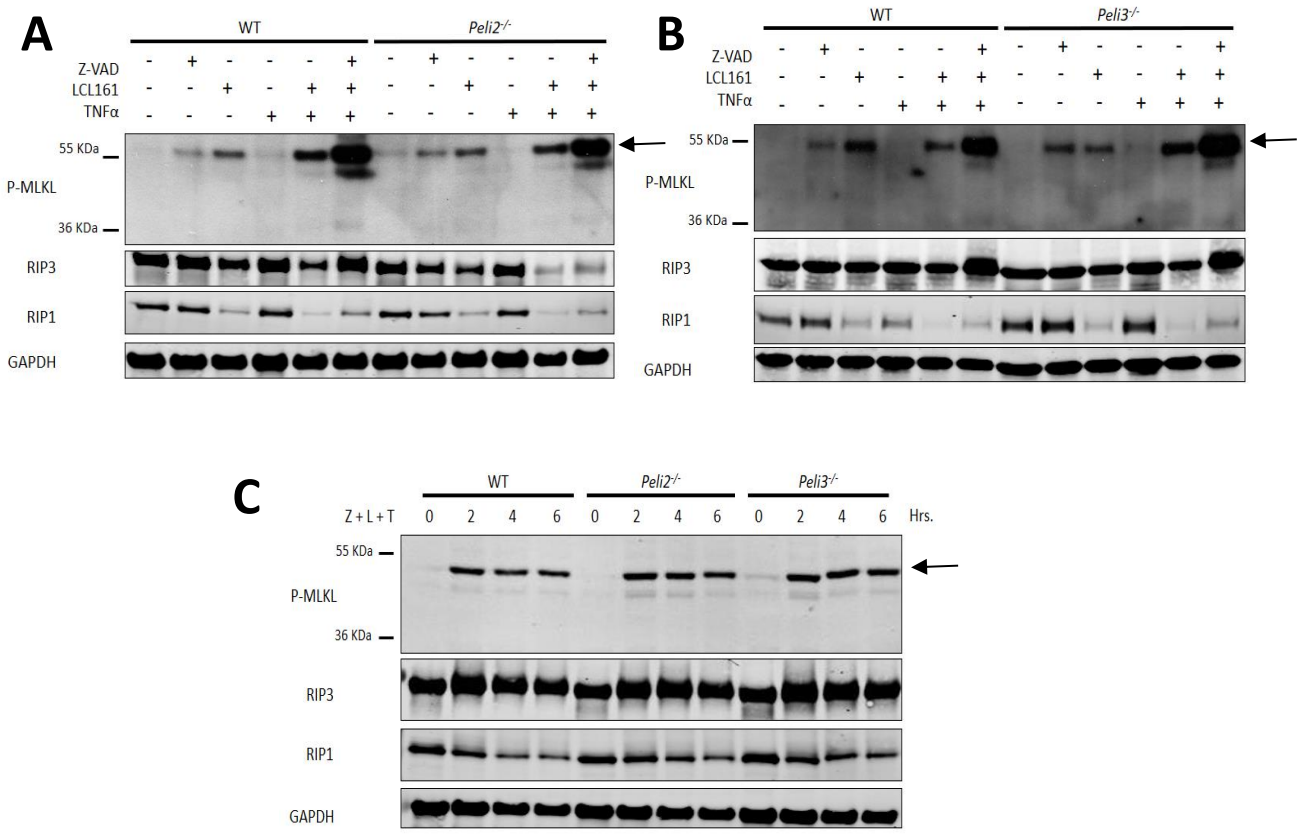

Figure 3.10 Effect of Pellino 2 or Pellino 3 deficiency on molecular markers of necroptosis induced with TNFα in BMDMs.

Immunoblot analysis of phosphorylated (p-) MLKL (54 kDa) and total levels of RIP1 (74 kDa) and RIP3 (46-53 kDa) in cell lysates from WT, *Peli2*$^{-/-}$ and *Peli3*$^{-/-}$ BMDMs pre-treated with 20 µM Z-VAD (Z) for an hour and stimulated with 10 µM LCL161 and 40 ng/mL TNFα (T) for the indicated times. Control of treatment at 6 hours in **(A)** WT and *Peli2*$^{-/-}$ BMDMs, and **(B)** WT and *Peli3*$^{-/-}$ BMDMs. **(C)** 2, 4 and 6 hours treatment time points in WT, *Peli2*$^{-/-}$ and *Peli3*$^{-/-}$ BMDMs. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

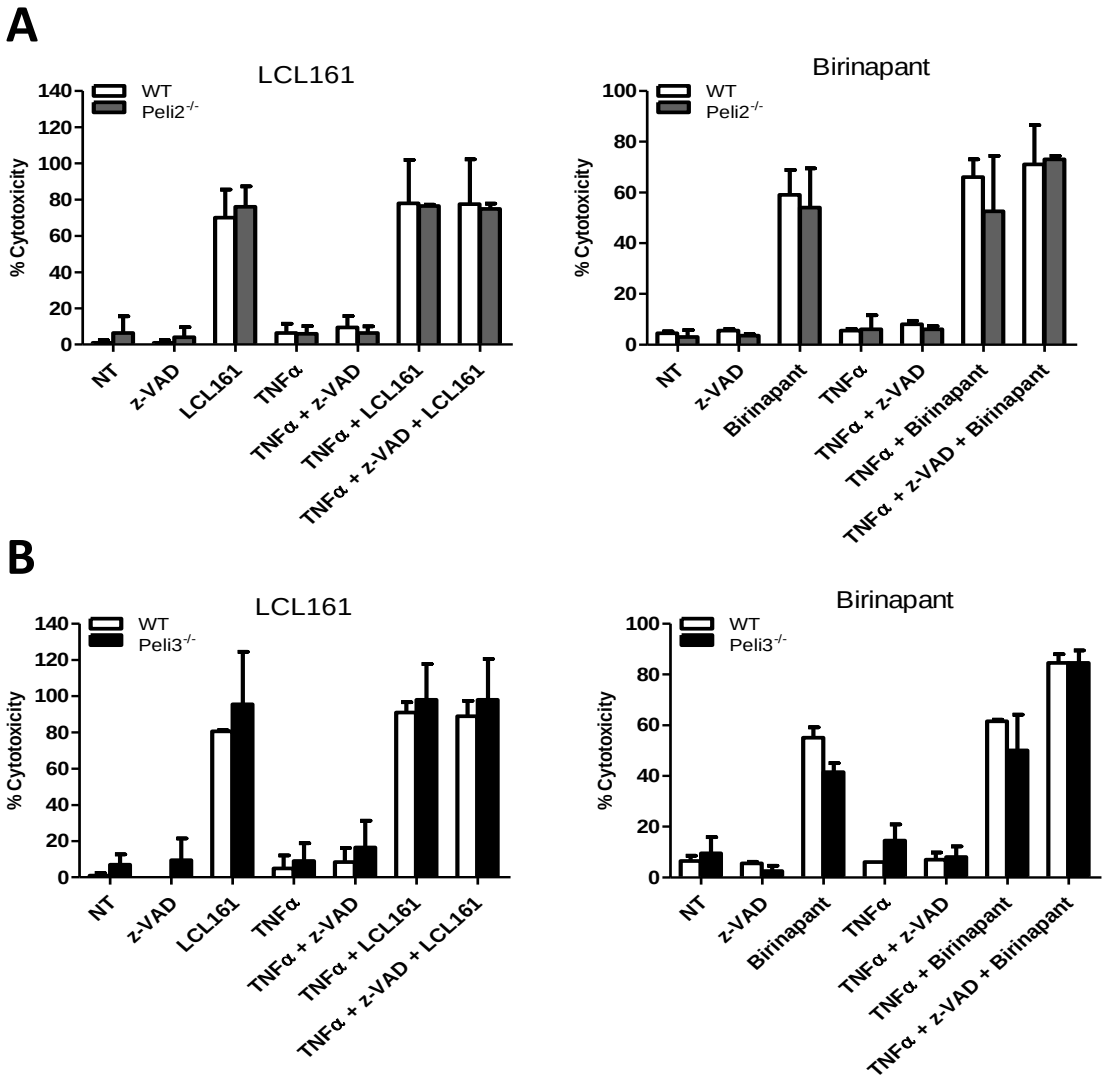

Figure 3.11 Pellino 2 or Pellino 3 deficiency does not affect TNF-induced necroptosis as measured by release of lactate dehydrogenase in BMDMs.

Cytotoxicity analysis by LDH release in supernatant from **(A)** WT and *Peli2*$^{-/-}$ BMDMs or **(B)** WT and *Peli3*$^{-/-}$ BMDMs pre-treated with 20 µM Z-VAD for an hour and stimulated with either 40 ng/mL TNFα and 10 µM LCL161 (left panels) or 40 ng/mL TNFα and 10 µM Birinapant (right panels) for 24 hrs. Data are presented as the mean ± SEM of two independent experiments. Maximum release of LDH was stablish for each biological replicate using lysis buffer provided with the LDL assay Kit. Additional details can be found in Section 2.2.2.3.

cytotoxicity when compared with WT BMDMs for treatment with LCL161 or its combined treatment with TNFα or Z-VAD and TNFα (**Figure 3.11B, left**). Finally, Birinapant increased cytotoxicity to 60% when used alone or combined with TNFα, and to 80% when used with Z-VAD and TNFα (**Figure 3.11B, right**). Nonetheless, the difference between WT and *Peli3*$^{-/-}$ BMDMs was very modest. This further support the earlier conclusion that neither Pellino 2 or Pellino 3 play any mediatory role in the process of necroptosis in macrophages.

Necroptosis induced by TNFα was also studied in dendritic cells (DCs), another important antigen presenting type of cell from the immune system. As described for the studies with macrophages, Pellino 2 deficient and WT DCs were treated with different combinations of Z-VAD, LCL161 and TNFα for 0-6 hours (**Figure 3.12**). Again, elevated levels of P-MLKL, slower migrating forms of RIP3 and reduced levels of RIP1 were apparent in response to the death-inducing signals but the same patterns were observed in Pellino 2 deficient cells and WT DCs suggesting a lack of role for these Pellino proteins in TNF-induced necroptosis in multiple cell types.

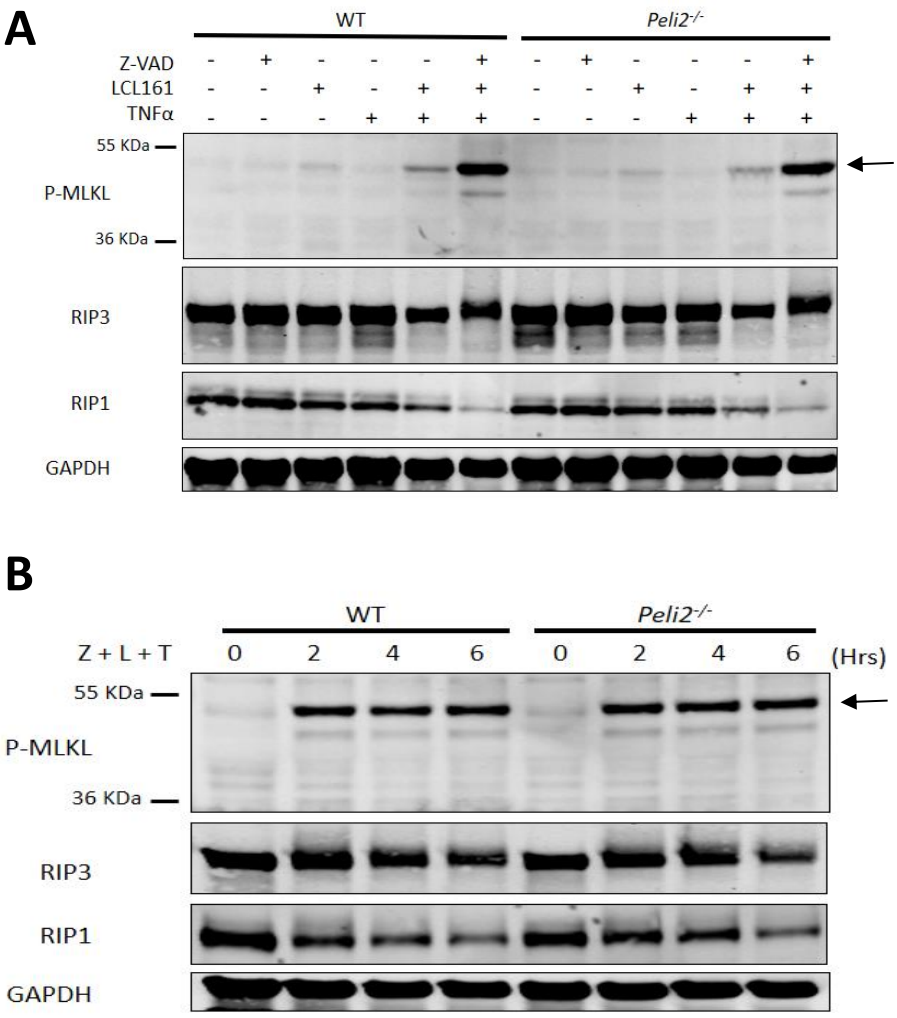

Figure 3.12 Effect of Pellino 2 deficiency on molecular markers of necroptosis induced with TNFα in dendritic cells.

Immunoblot analysis of phosphorylated (p-) MLKL (54 kDa) and total levels of RIP1 (74 kDa) and RIP3 (46-53 kDa) in cell lysates from WT and *Peli2*$^{-/-}$ DCs pre-treated with 20 µM Z-VAD (Z) for an hour and stimulated with 10 µM LCL161 (L) and 40 ng/mL TNFα (T) for the indicated times. **(A)** Control of individual treatments at 6 hours, and **(B)** 2, 4 and 6 hours treatment time points. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

3.2.3 Effect of Pellino 2 and Pellino 3 deficiency in necroptosis induced by TLR4 and TLR3 in murine macrophages

Whilst Pellino 2 and Pellino 3 does not appear to play a role in TNF-induced necroptosis, the next studies aimed to explore their potential role in TLR-induced cell death, especially given their previously reported roles in TLR biology. Indeed, cell death ligands like TNFα are not the only way to activate necroptosis. Necroptosis can also be triggered by Lipopolysaccharide (LPS), a main component of Gram-negative bacteria membranes, that acts as ligand for TLR4. TLR4-induced necroptosis does not depend directly on RIP1/RIP3 interaction but instead is mediated by the interaction between TRIF and RIP3 (He *et al.*, 2011; Kaiser *et al.*, 2013). As in previous experiments, Pellino 2 deficient and WT BMDMs were pre-treated for 1 h with the pancaspase inhibitor Z-VAD and treated with LCL161 and the TLR4 ligand LPS for 6 or 24 hours. Strong phosphorylation of MLKL was apparent in WT BMDMs cells in response to 6 hours treatment with all 3 agents (**Figure 3.13A**). Phosphorylation of the necroptosis effector proteins was also faintly induced by Z-VAD alone. The levels of RIP1 decreased when cells were treated with LPS and Z-VAD in the absence and presence of LCL161 and with some retardation of the electrophoretic mobility of RIP3 when cells were treated with the pro-necroptotic treatment regime of all 3 agents. The same patterns of P-MLKL, RIP1 and RIP3 were also apparent in Pellino 2 deficient BMDMs. Similar findings were observed at treatment periods of 24 h with an additional reduction of RIP1 and RIP3 when inhibitors Z-VAD and LCL161 were used (**Figure 3.13B**). These studies indicate that Pellino 2 is not employed by the TLR4 pathway in triggering necroptosis in macrophages.

Equivalent studies were also performed in WT and *Peli3*$^{-/-}$ BMDMs to explore the role of Pellino 3 in LPS-induced necroptosis and the patterns of MLKL phosphorylation and changes in levels of RIP1 an RIP3 were the same in cells from both mice (**Figure 3.14**). Together these data indicate that neither Pellino 2 or Pellino 3 mediate necroptosis that is induced by TLR4 signalling. Given the previously described role for Pellino proteins in TLR3 signalling coupled to the role of TLR3 in triggering necroptosis the next study explored the role of Pellino 2 and Pellino 3 in TLR3-induced necroptosis (Chang, Jin and Sun, 2009; Siednienko *et al.*, 2012). Poly(I:C), a ligand for TLR3, was used in absence or presence of the pancaspase inhibitor Z-VAD for 6 or 18 hours. Necroptosis molecular markers were measured in Pellino 2/Pellino 3 deficient BMDMs and compared with WT cells. Phosphorylation of MLKL was faintly seen at 6 hours for all the phenotypes but there was no consistent change between cells from WT and Pellino-deficient mice (**Figure 3.15A**). No obvious variations were observed for RIP3 and RIP1 at this time point. Treatment of cells with Poly(I:C) and Z-VAD for 18h promoted the appearance of high levels of

P-MLKL with comparable levels being apparent in WT, Pellino 2- and Pellino 3-deficient BMDMs (**Figure 3.15B**) again supporting the absence of roles for Pellino 2 and Pellino 3 in necroptosis.

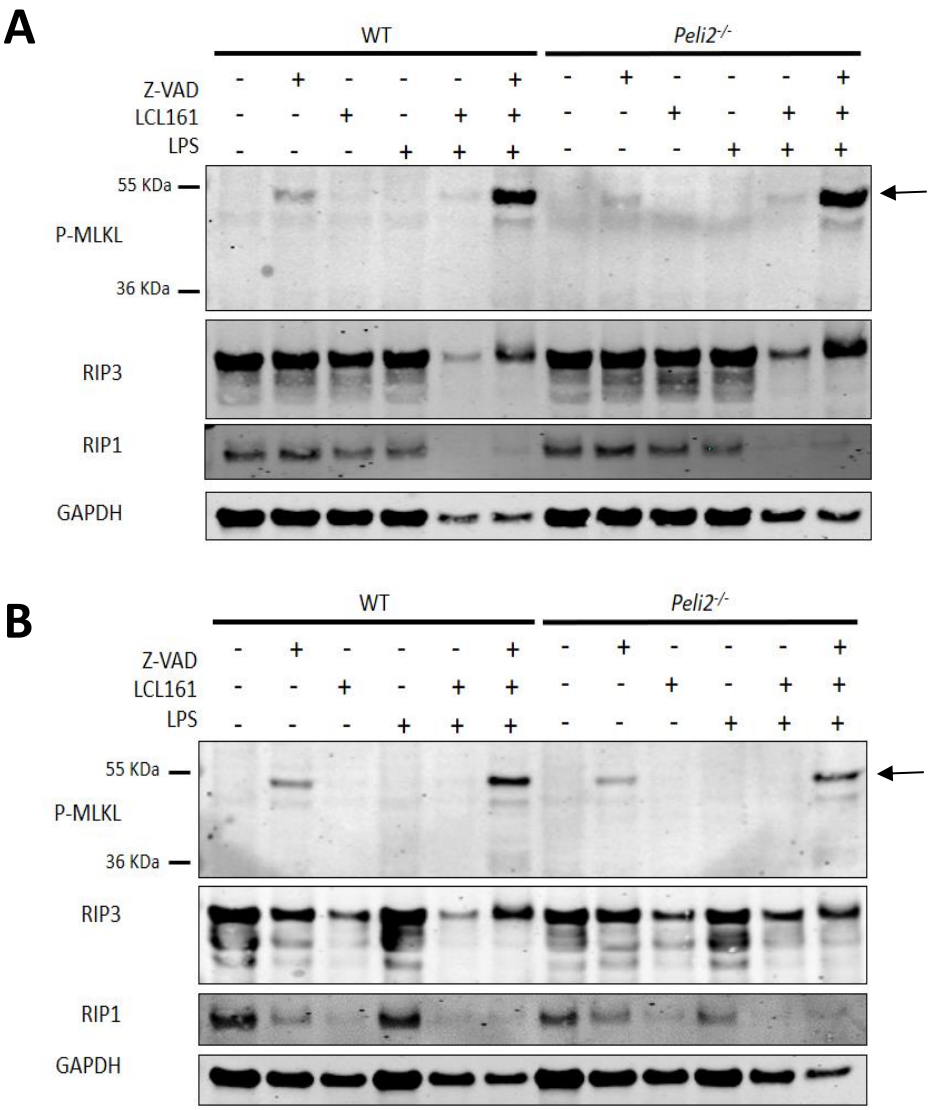

Figure 3.13 Effect of Pellino 2 deficiency on molecular markers of necroptosis induced with LPS in BMDMs.

Immunoblot analysis of phosphorylated (p-) MLKL (54 kDa) and total levels of RIP1 (75 kDa) and RIP3 (46-53 kDa) in cell lysates from WT and *Peli2*$^{-/-}$ BMDMs pre-treated with 20 µM Z-VAD for an hour and stimulated with 10 µM LCL161 and 40 ng/mL LPS for **(A)** 6 hrs and **(B)** 24 hrs. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

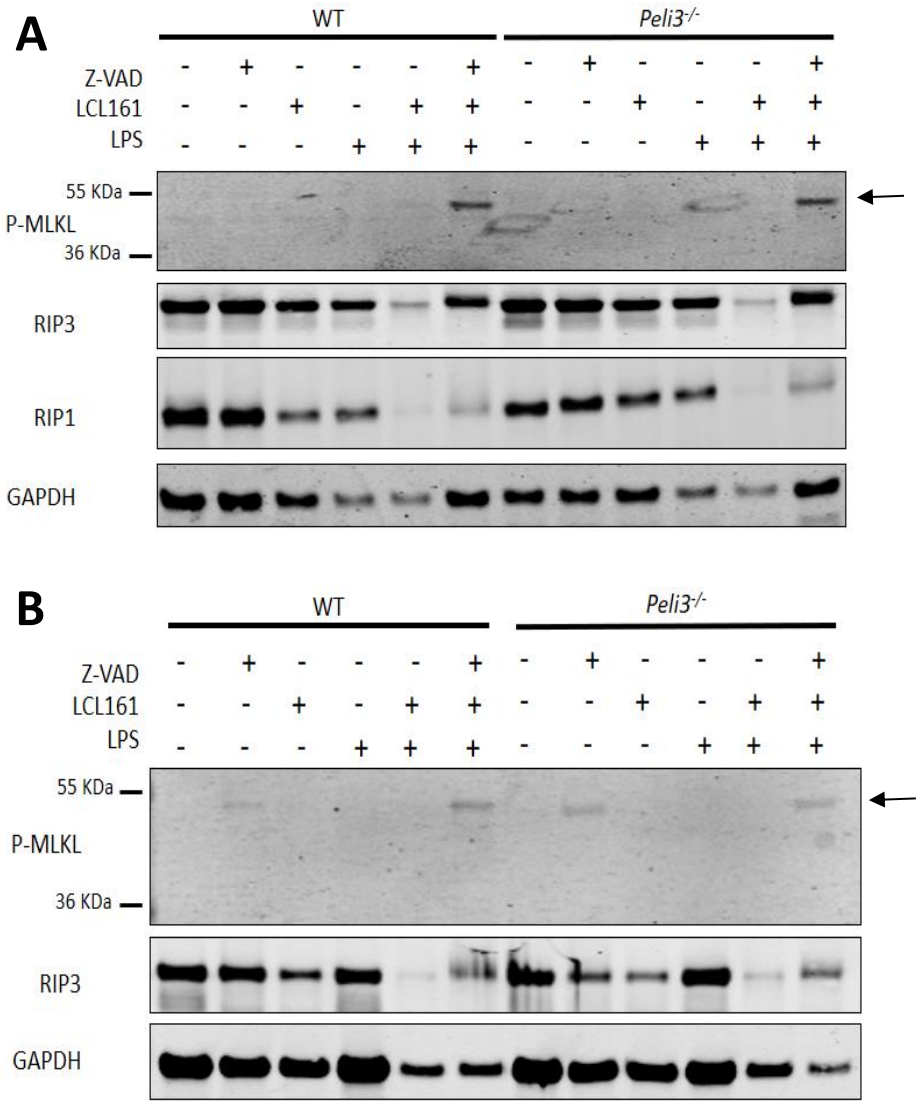

Figure 3.14 Effect of Pellino 3 deficiency on molecular markers of necroptosis induced with LPS in BMDMs.

Immunoblot analysis of phosphorylated (p-) MLKL (54 kDa) and total levels of RIP1 (74 kDa) and RIP3 (46-53 kDa) in cell lysates from WT and *Peli3*$^{-/-}$ BMDMs pre-treated with 20 µM Z-VAD for an hour and stimulated with 10 µM LCL161 and 40 ng/mL LPS for **(A)** 6 hrs and **(B)** 24 hrs. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

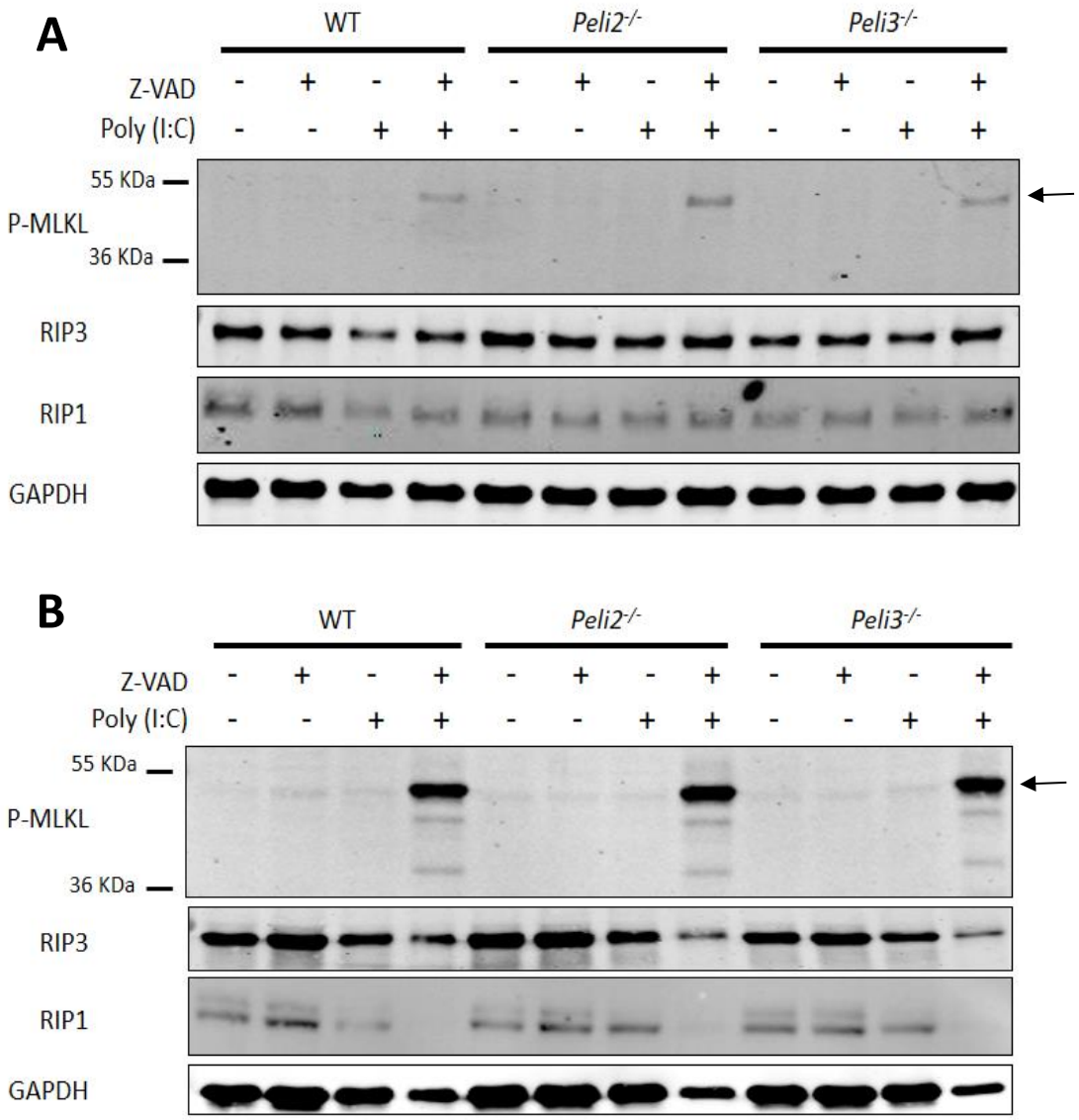

Figure 3.15 Effect of Pellino 2 or Pellino 3 deficiency on molecular markers of necroptosis induced with Poly(I:C) in BMDMs.

Immunoblot analysis of phosphorylated (p-) MLKL (54 kDa) and total levels of RIP1 (74 kDa) and RIP3 (46-53 kDa) in cell lysates from WT, *Peli2*$^{-/-}$ and *Peli3*$^{-/-}$ BMDMs pre-treated with 20 µM Z-VAD for an hour and stimulated with 25 µg/mL Poly(I:C) for **(A)** 6 hrs and **(B)** 18 hrs. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

3.3 Discussion

Necroptosis is a lytic type of regulated cell death described nearly ten years ago (Galluzzi and Kroemer, 2008; Hitomi *et al.*, 2008). Our understanding of its molecular mechanism and regulation has increased since then. So far, it is clear the family of RIP kinases, especially RIP1 and RIP3, play essential roles as triggers and mediators in this type of cell death (Cho *et al.*, 2009; Li *et al.*, 2012; Vanden Berghe *et al.*, 2014; Wu *et al.*, 2014; Almagro and Vucic, 2015). RIP1 is a kinase that relies on ubiquitination to be activated and mediate pro-inflammatory or cell death signalling. Work from our laboratory and other researchers reported that the Pellino family of E3 ligases can regulate RIP kinase function in both inflammation and cell death (Chang, Jin and Sun, 2009; Yang, Wang, Humphries, *et al.*, 2013; Yang, Wang, Tang, *et al.*, 2013). Therefore, the role of Pellino E3 ligases in the regulation of necroptosis was explored. Inhibition of both the inflammatory and apoptotic signalling pathways by means of cIAP/XIAPs and caspases, respectively, allowed to drive the signalling cascades toward necroptosis when MEFs, BMDMs or DCs were stimulated with death receptor ligand TNFα. A number of molecular readouts were employed as indices of necroptosis. Immunoblotting of three critical proteins (MLKL, RIP3 and RIP1) involved in the necroptotic cascade were chosen as the best approach. Other techniques such as AnnexinV/PI staining and percentage of cytotoxicity by LDH release have also been reported as possible read outs of necroptosis but late apoptosis and other types of lytic cell death like pyroptosis can also increase these parameters (Galluzzi *et al.*, 2014). A clear example of this was apparent in MEFs treated with Z-VAD, different inhibitors of IAPs (BV6, LCL161 and Birinapant) and TNFα stimulation when cytotoxicity was evaluated by means of LDH release (**Figures 3.7 and 3.11**). Treatment of cells with IAP inhibitors and TNFα, which were driving the cell to apoptosis, showed a similar percentage of cytotoxicity compared with cells stimulated with Z-VAD, IAP inhibitors and TNFα, that drive cells towards necroptosis. Hence, immunoblotting of MLKL, RIP1 and RIP3 were used as the preferred markers of activation of the necroptotic pathway.

Phosphorylated MLKL was apparent across a number of cell types when stimulated with TNFα, Z-VAD and LCL161. These results are consistent with the working model of necroptosis signalling, where TNFR-dependant activation triggers phosphorylation of the effector molecule MLKL when the inflammatory and apoptotic signalling pathways are blocked (Sun *et al.*, 2012; Vanden Berghe *et al.*, 2014; Rodriguez *et al.*, 2016). P-MLKL was also present to a minor extent when IAPs inhibitors were used with TNFα stimulation. Expression in this situation was much

lower compared with necroptotic conditions that blocked caspase activity. Whilst, under conditions of co-stimulation with IAP inhibitors and TNFα, cells die primarily by apoptosis, a small proportion seems to take the necroptotic fate and it is interesting to speculate that this may represent secondary necroptosis in response to primary apoptosis. The use of pan-caspase inhibitors could be driving the cells which would spontaneously die by apoptosis towards necroptosis, or its prolonged use could be generating intracellular stress in a reduced number of cells in cell culture thus triggering necroptosis (Vanden Berghe *et al.*, 2014; Shalini *et al.*, 2015). In a similar way, treatment with IAP inhibitors alone clearly induces cell death, as seen with the increase in cytotoxicity in both MEFs and BMDMs when BV6, LCL161 or Birinapant were used alone (**Figures 3.2.2 and 3.2.6**). However, LDH release does not allow to define the type of cell death. Immunoblot analysis indicates that a small proportion of cells died by necroptosis when antagonists of IAPs were used alone, and such an effect was more prominent with the use of LCL161 compared with BV6. The autoubiquitination of IAPs and subsequent degradation in response to IAP inhibitors sensitises mammalian cells to apoptosis (Vasudevan and Ryoo, 2015). However, our results also showed a moderate activation of the necroptotic pathway in particular for LCL161, which has been reported to have high affinity for both cIAP and XIAPs (Lawlor *et al.*, 2015). Interestingly, loss of XIAP or inactivation of its RING domain has been shown to decrease cell survival in a RIP3 dependant way in murine bone marrow derived dendritic cells (BMDCs) (Yabal *et al.*, 2014). In the light of those findings and the results of this thesis, a potential regulation of RIP3 and downstream activation of necroptosis mediated by XIAP could be addressed in future studies.

RIP3 and RIP1 expression was also evaluated in the present studies. Interestingly, when the cells were directed toward necroptosis, a slight increase in the size of RIP3 was observed, as evidenced by reduced electrophoretic mobility. The upward shift of RIP3 band seems to reflect its phosphorylation state characteristic of necroptosis signalling (Cho *et al.*, 2009; He *et al.*, 2009; Zhang *et al.*, 2009; Newton *et al.*, 2014; Alturki *et al.*, 2018). In addition, when MEFs, BMDMs or DCs are driven towards apoptosis by IAP inhibitors and TNFα or TLR4 stimulation, RIP3 expression is moderately reduced at 6 hours and more clearly lost at longer time points. It has been described that active caspase 8 cleaves RIP3 at Asp328 in the context of apoptosis (Feng *et al.*, 2007). As expected, the levels of RIP1 were also reduced in apoptosis given it is degraded by caspase-8 under pro-apoptotic conditions (Lin *et al.*, 1999; Chan *et al.*, 2003). RIP1 was likewise considerably reduced or absent when cells were driven to necroptosis. However, it was not clear whether this was due to degradation of RIP1 induced after auto-phosphorylation of RIP3 and induction of its homo-interacting structure (Wu *et al.*, 2014). Alturki and co-workers

(2018) reported recently the proteasomal degradation of RIP1 mediated by the E3-ubiquitin ligase Triad3a in macrophages stimulated with LPS in the presence of Z-VAD. They described a K48-ubiquitin-dependent proteasomal degradation of this kinase in early necrosome formation evident after 2 hours of stimulation. Interestingly, this research suggests that once necroptosis is initiated, the interaction between RIP3 and RIP1 mediates the degradation of the latter (Alturki *et al.*, 2018). Such findings add to the complex interaction between these two kinases and their dual loop of regulation, since whilst RIP1 mediates formation of the necrosome with RIP3, it can also suppress basal activation of RIP3 (Kearney *et al.*, 2014; Orozco *et al.*, 2014). Evidently our model of necroptosis and the expression of the three main proteins associated with it fit consistently with the working model of this type of regulated cell death.

The early studies in this thesis investigated whether E3 ligases Pellino 2 and 3 have a role in regulation of necroptosis. We found that loss of Pellino 2 and/or Pellino 3 had modest or no difference in the expression of the necroptosis markers evaluated across three types of cells (MEFs, BMDMs or DCs). A very modest decrease in the band for phosphorylated MLKL was seen in Pellino 2 deficient MEFs when compared with WT MEFs in the context of TNFα death receptor signalling. Interestingly, no difference was seen for the same protein in BMDMs or DCs when comparing Pellino 2-deficient cells with WT cells when stimulated with TNFα. However, there was a slight increase in P-MLKL in Pellino 2 deficient BMDMs compared with WT cells when necroptosis was induced via TLR signalling. The discreet increase in the band was seen at 6 hours treatment for both LPS and Poly(I:C) and corresponded to a similarly discreet increase in RIP3, but no evident change in RIP1. Interestingly, it seems the change is reversed or lost at longer time points for both ligands of TLR4 and TLR3 respectively. These results could suggest a minor role of Pellino 2 in regulating TRIF-dependant activation of the necrosome perhaps directly affecting RIP3 in early stages of the process. Another member of the Pellino family has been shown to regulate RIP1 in the context of TRIF-dependant signalling. TLR4 and TLR3 induced activation of NF-κB, which was shown to depend on Pellino 1-induced ubiquitination of RIP1 (Chang, Jin and Sun, 2009). Furthermore, the same member of the family was recently described to modulate necroptosis signalling by regulating both kinases, RIP1 and RIP3 (Wang *et al.*, 2017; Choi *et al.*, 2018). Using a similar approach to the one in this thesis, Wang and collaborators treated WT and Pellino 1-deficient MEFs with TNFα, Z-VAD and SM164, an inhibitor of cIAPs and XIAPs, and demonstrate that Pellino 1 induces K63 ubiquitination of RIP1 on K115. The authors concluded that ubiquitination of RIP1 by Pellino 1 is indispensable for the interaction of this kinase with RIP3 and the subsequent formation of the necrosome and initiation of necroptosis. It is to note that recognition of RIP1 by Pellino 1 seems to depend on RIP1 phosphorylation status

(Wang *et al.*, 2017). In contrast, earlier this year, Choi and co-workers reported that Pellino 1 is an important inhibitor of necroptosis, by mediating the degradation of RIP3 (Choi *et al.*, 2018). The authors showed that phosphorylated RIP3 is recognized by the FHA domain of Pellino 1 which mediates K48 polyubiquitination of the kinase on its K363 residue. They too describe proteasomal degradation of this targeted RIP3 and downregulation of necroptosis. Furthermore, this study describes a correlation between increased expression of RIP3 and low levels of Pellino 1 in keratinocytes from toxic epidermal necrolysis (TEN) patients, and suggests that the increase in necroptosis due to the lack of Pellino 1 could be the mechanism explaining the pathogenesis of this disease (Choi *et al.*, 2018). The basis for these conflicting reports on the role of Pellino 1 in necroptosis is unknown. Studies in this thesis extends to an investigation into the role of the other two Pellino family members, Pellino 2 and Pellino 3 and all of our data are consistent with a lack of involvement for Pellino 2 and Pellino 3 in necroptosis across a number of cell type. This highlights a lack of functional redundancy for members of the Pellino family with Pellino 1 likely playing some regulatory function in necroptosis whereas the other two-family members lack of effect. This may be surprising since Pellino members are very similar in terms of primary structure and predicted secondary structure. Each member has a common FHA and RING-like domains. However, they appear to be functionally unique based on reports to date. In that sense, Pellino 1 has been described to promote TLR3 and TLR4-activation of NF-κB by polyubiquitination of RIP1 in a RING-domain dependent mechanism, while Pellino 3 has been shown to interact with RIP1 through its FHA domain preventing the initiation of apoptosis (Chang, Jin and Sun, 2009; Yang, Wang, Tang, *et al.*, 2013). Other variable roles have been described for Pellino 1 and Pellino 3 in the regulation of IL-1 signalling, where Pellino 1 is recognized as a positive regulator of IL-1 while Pellino 3 seems to negatively regulate the same pathway (Jiang *et al.*, 2003; Xiao *et al.*, 2008). Less is known about the function of Pellino 2, however, research from our laboratory has proven that it has an essential role in the regulation of activation of the NLRP3 inflammasome through both the FHA and RING-like domains (Humphries *et al.*, 2018). Under circumstances of NLRP3 inflammasome activation, Pellino 2 also plays a mediatory role in a form of necrotic cell death known as pyroptosis. However, data described above clearly show that neither Pellino 2 or Pellino 3 regulate necroptosis, another form of cell necrosis. These findings clearly highlight the selectivity and specificity of Pellino action in different cell pathways and processes in innate immune signalling.

Whilst Pellino 2 and Pellino 3 appear to lack a role of necroptosis in the context of inflammatory stimuli, our research group has previously demonstrated an important role for Pellino 3 in controlling the transcription factor HIF-1α (Yang *et al.*, 2014). However, the latter

has been largely studied in the context of a response factor to low levels of oxygen. This thesis next moves to a broader evaluation of the ability of innate immune stimuli to affect HIF-1α.

CHAPTER FOUR
HIF-1α AND INFLAMMATION

4.1 Introduction

Hypoxia is a state of low concentration of oxygen that damages tissue homeostasis. It is triggered when physiological, mechanical or external conditions decrease O_2 levels, and it can happen at cellular, tissue or systemic levels. A considerable number of genes are induced under these conditions aiming to reduce the negative impact of cellular oxygen stress (Lisy and Peet, 2008). The hypoxia-inducible transcription factors (HIFs) are the key proteins orchestrating this complex response which includes several cellular processes to regulate oxygen dependence and consumption, and the co-ordination of effective oxygen delivery to cells (Lisy and Peet, 2008). Vascularization, vasodilation, cell migration, metabolism, signalling and cell fate decisions are some of the mechanisms altered by HIF signalling (Iyer, Leung and Semenza, 1998; Lisy and Peet, 2008). Considering the influence of HIF proteins in such fundamental processes, they have also been shown to play a role in the pathophysiology of some human diseases such as cancer, coronary artery disease, rheumatoid arthritis, stroke, and inflammatory bowel disease among others (Iyer, Leung and Semenza, 1998; Scholz and Taylor, 2013; Balamurugan, 2016; Cummins *et al.*, 2016). Research from our group has demonstrated a novel role for Pellino 3 in negatively regulating HIF signalling, and so protect against obesity-induced inflammation and insulin-resistance (Yang *et al.*, 2014). Whilst HIF has traditionally been studied in the context of oxygen sensing there is a growing interest in its role in regulating inflammation and the main goal of this chapter was to explore the potential regulatory effect of TLR signalling on HIF activation.

4.1.1 HIF structure and signalling

The functional activity of HIF proteins depends on the assembly of a heterodimer comprising an α and β HIF subunits (Wang and Semenza, 1995; Wang *et al.*, 1995). Both subunits contain a transactivation domain and a DNA binding domain which belongs to the basic helix–loop–helix/Per-Arnt-Sim homology (bHLH/PAS) family of transcription factors (**Figure 4.1**) (Jiang, Rue, *et al.*, 1996; Bracken, Whitelaw and Peet, 2003; Chapman-Smith and Whitelaw, 2006). The stability and transcriptional activity of the HIF-α subunit are regulated by oxygen levels while HIF-β is constitutively expressed in the nuclei (Wang *et al.*, 1995; Jiang, Semenza, *et al.*, 1996). Both subunits have paralogues. HIF-α subunit can be found as HIF-1α, HIF-2α and HIF-3α, while HIF-β (also known as aryl hydrocarbon receptor nuclear translocator (Arnt), is found in the subtypes Arnt1, Arnt2 and Arnt3 (Gu *et al.*, 1998; Semenza, 1999; Lisy and Peet, 2008). HIF-1α

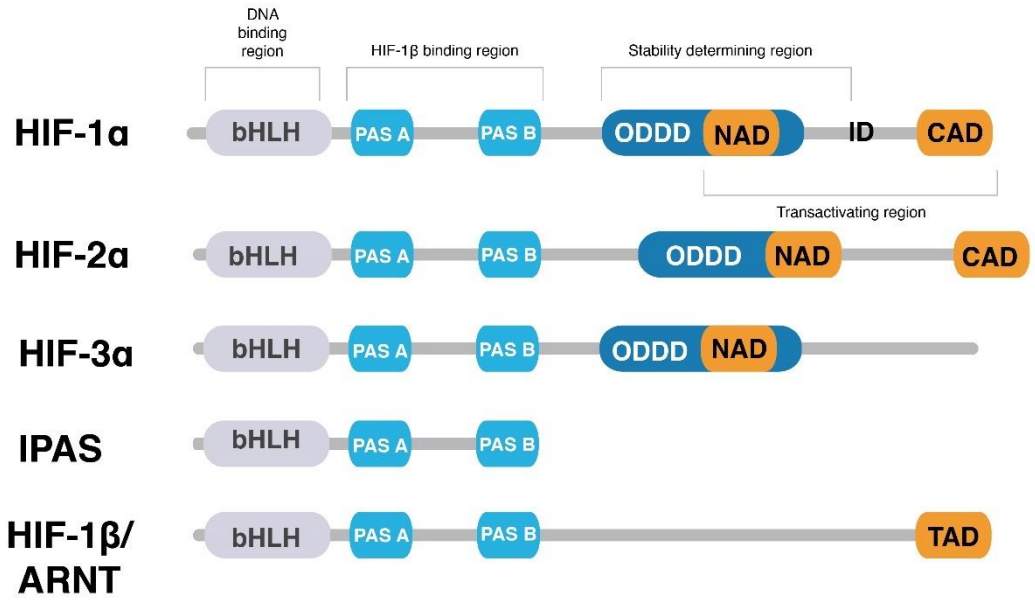

Figure 4.1 Hypoxia-inducible transcription factors (HIFs) structural domains.

Functionally active HIF is a heterodimer formed by two subunits known as α and β. There are three paralogues of HIFα named HIF-1α, HIF-2α and HIF-3α. In addition, a spliced form of HIF3α recognized as IPAS has been described. HIF-1α forms a dimer with HIF-1β also known as ARNT. All of them have bHLH and PAS domains in common which allow for DNA recognition and binding. In addition, the three HIFα isoforms contain ODDDs, NADs and CADs domains, which mediate stabilization and transactivation of HIF proteins (Adapted from Lisy & Peet, 2008).

and HIF-2α have been described to interact with HIF-1β to form the functional HIF transcription factor complexes responsible for the hypoxic response (Semenza, 1999; Lisy and Peet, 2008). In contrast, HIF-3α and its alternative spliced form known as inhibitory PAS domain protein (IPAS) have been described as negative regulators of HIF-mediated transcription (Gu *et al.*, 1998; Makino *et al.*, 2001). IPAS has been shown to dimerise with HIF-1β, sequestrating HIF-1β and interfering with its binding to HIF-1α (Makino *et al.*, 2001). The carboxyl-terminal portion of HIF-1α includes stabilizing and transactivation domains which determines its oxygen-dependant stabilization and transcriptional activity, while its amino-terminal portion contains bHLH/PAS domains that allow for heterodimerization with HIF-1β and DNA binding (Jiang, Rue, *et al.*, 1996; Jiang, Semenza, *et al.*, 1996; Jiang *et al.*, 1997; Pugh *et al.*, 1997).

HIF-1α is constitutively transcribed, translated and constantly degraded within the cells under normoxia conditions (Huang *et al.*, 1996; Yu *et al.*, 1998). Its degradation in less than 5 minutes is dependent on initial hydroxylation of two conserved prolyl residues within the central oxygen-dependent degradation domain (ODDD) in the C-terminal portion of the protein (**Figures 4.1 and 4.2**) (Semenza, 1999; Lisy and Peet, 2008). HIFα hydroxylation is mediated by prolyl hydroxylases (PHDs) and it allows for HIFα recognition and polyubiquitination by the von Hippel-Lindau protein (pVHL) (Maxwell *et al.*, 1999; Semenza, 2004). Ubiquitinated HIFα becomes a target for proteasomal degradation (**Figure 4.2**). However, under hypoxic conditions, PHD hydroxylation activity is hampered (as it requires oxygen as a substrate) allowing for HIFα stabilization and translocation to the nucleus (Semenza, 2004). Increased stability of HIFα can also be achieved by its binding to heat-shock protein 90 (Hsp90) in an oxygen-independent way (Liu and Semenza, 2007). In addition to hydroxylation by PHDs, another member of the dioxygenase family of hydroxylases named factor-inhibiting HIF-1α (FIH-1) plays an important role HIF signalling. FIH-1 induces the hydroxylation of a single conserved asparagine residue in the C-terminal CAD domain under normoxia conditions (D. Lando *et al.*, 2002; David Lando *et al.*, 2002; Hewitson *et al.*, 2002). Hydroxylation of this asparagine residue in position 803 inhibits HIFs transcriptional activity by blocking the interaction of its CAD domain with the essential transcriptional coactivator CREB-binding protein (CBP) or p300 (David Lando *et al.*, 2002; Hewitson *et al.*, 2003). Considering both types of hydroxylase enzymes require oxygen to exercise their enzymatic activity, they act as cellular oxygen sensors regulating HIF-1α degradation and functional activity under normoxic conditions (Semenza, 2004; Lisy and Peet, 2008).

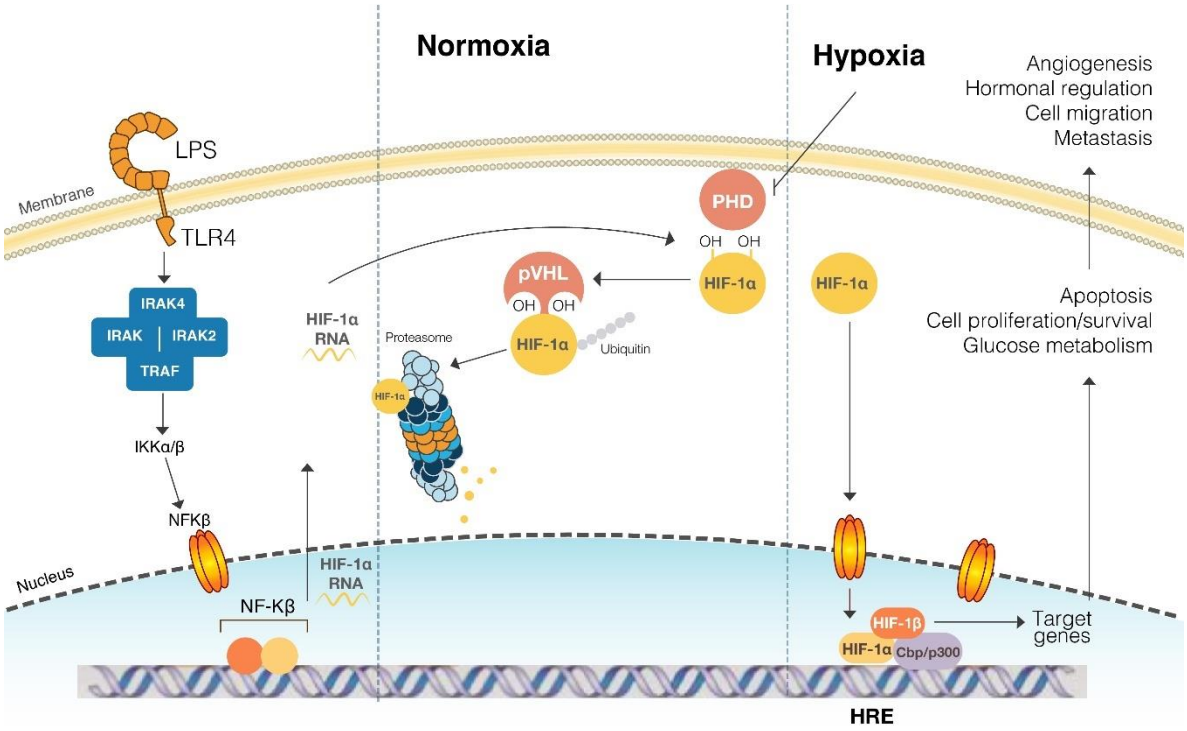

Figure 4.2 HIF transcriptional regulation.

HIFα is constantly been produced and degraded under normoxia conditions. At normal levels of oxygen, HIFα is hydroxylated by enzymes PHD and FIH that target the protein for degradation and blocks its transactivation function respectively. Once hydroxylated, VHL protein can recognize HIFα and mediate its polyubiquitination and subsequent degradation by the proteasome. In hypoxia, hydroxylase enzymes are inhibited, HIFα can then translocate to the nucleus and form a dimer with HIFβ. The heterodimer binds to the HRE sequence in its target genes and recruit cofactors like CBP and P300 that will facilitate the transcription of the corresponding genes. HIF-1α has also been described to accumulate in response to bacteria and bacterial products such as LPS in a signalling cascade dependent on NF-κB. *CBP, CREB-binding protein; FIH, factor inhibiting HIF; HIF, hypoxia-inducible factor; HREs, HIF-responsive elements; LPS, lipopolysaccharide; P300, E1A binding protein; PHD, prolyl hydroxylase domain-containing proteins; VHL, von Hippel-Lindau*.

When oxygen concentrations drop, HIF-1α hydroxylation is blocked, it is then translocated to the nucleus where it dimerises with HIF-1β through their common bHLH/PAS domains (Jiang, Rue, *et al.*, 1996; Jiang, Semenza, *et al.*, 1996; Jiang *et al.*, 1997; Pugh *et al.*, 1997). This domain recognizes the hypoxia response elements (HREs) in the promotor of over 70 genes (Liu *et al.*, 2012; Dengler, Galbraith and Espinosa, 2014). Two regions are recognized in the genes, a core HRE or HIF-binding site (HBS) containing the sequence (A/G)CGTG and the HIF ancillary sequence (HAS) (Semenza and Wang, 1992; Kimura *et al.*, 2001). After its assembly on the HRE, HIF forms an initiation complex by recruiting transcriptional co-activators through its transactivation domains (Lisy and Peet, 2008). The oxygen-regulated C-terminal transactivation domain (CAD) and the N-terminal transactivation domain (NAD) are the portions of HIF-1α and HIF-2α responsible for recruiting coactivators CBP/p300, SRC-1, and transcription intermediary factor 2 (TIF-2) (Arany *et al.*, 1996; Jiang *et al.*, 1997; Pugh *et al.*, 1997; Ema *et al.*, 1999; D. Lando *et al.*, 2002). These two transactivation domains of HIF-1α and HIF-2α have been shown to be the key modulators of HIF target genes and their activity has been reported to be regulated by oxygen levels, especially CAD (Jiang *et al.*, 1997; Dayan *et al.*, 2006). Nevertheless, a small number of HIF target genes seem to be dependent solely on NAD activity suggesting that these two domains are regulators of specific responses to low concentrations of oxygen (Dayan *et al.*, 2006; Hu *et al.*, 2007). The CAD domain is also present in HIF-1β but contrary to its role in HIF-1α and HIF2α is believed not to be necessary for HIF transcriptional activation (Bracken, Whitelaw and Peet, 2003). CBP/p300 has been described as the main coactivator associated with the HIF heterodimer. It allows HIF binding to other coactivator complexes and its histone acetyltransferase activity facilitating the chromatin modifications required for gene transcription (Arany *et al.*, 1996). HIF transcriptional activation regulates multiple cellular processes including angiogenesis, oxygen supply, proliferation, stemness/self-renewal, epithelial to mesenchymal transition (EMT), redox homeostasis, apoptosis and others (**Table 4.1**) (Iyer, Leung and Semenza, 1998; Liu *et al.*, 2012; Dengler, Galbraith and Espinosa, 2014). The switch between oxidative phosphorylation and glycolysis is one of the main cellular pathways influenced by HIF signalling (Dengler, Galbraith and Espinosa, 2014). HIF-mediated variations in glucose metabolism, cell migration, survival and angiogenesis have been shown to favour the development of solid tumours and the progression of several other diseases, including kidney-disease-related anaemia, cardiac ischaemia, fatty liver and diabetes (Wu *et al.*, 2015).

Cellular processes and disease processes regulated by HIF transcription

Angiogenesis and oxygen supply	ADM	EDN1	NOX2
	ANGPT1	EPO	NOS2/3
	ANGPT2	PGF	VEGF
	ANP	FLK1	GPI
	PDGFβ	CXCL12	HMOX1
Apoptosis	BNIP3/3L	PP5	NDRG
	NOXA	MCL1	NPM
Cell migration	CXCR4	c-MET	
Cell proliferation and survival	CD73	IGFBP3	EDD1
	CTGF	ITF	STK15
	TGFβ3		
Epithelial to mesenchymal transition (EMT)	ID2	SNAI1/2	TCF3
	TGF2	VIM	ZEB1/2
Glucose metabolism/energy metabolism	ALDA	LDHA	DEC1
	GLUT1/3	PFKL	PDK1
	GAPDH	PGK1	BNIP3(L)
	COX412	PKM2	
Hormonal regulation	EPO	LEP	
Metastasis	AMF	LOX	STC2
	ANGPTL4	LOXL2	TWIST1
	CXCL12	LOXL4	CTSC
	CXCR4	L1CAM	MMP1/2/9/14
pH regulation	CA9	CA12	
Redox homeostasis	GPX3	HMOX1	SOD2

Table 4.1 Cellular processes regulated by HIFs transcriptional activation and its target genes.

The list of HIF target genes is illustrative but not complete. *ADM, Adrenomedullin; ALDA, Aldolase A; AMF, Autocrine motility factor; ANGPTL4, Angiopoietin-like protein 4; ANGPT1/2, Angiopoietin-1/2; ANP, Atrial natriuretic peptide; BNIP3(L), BCL2 Interacting Protein 3; CA9/12, Carbonic anhydrase 9/12; CD73, Ecto-5'-nucleotidase; COX412, cytochrome c oxidase subunit 4I2; CTGF , Connective tissue growth factor; CTSC, Cathepsin C; CXCL12, C-X-C motif chemokine 12; CXCR4, C-X-C chemokine receptor type 4; c-MET, Mesenchymal-epithelial transition factor; DEC1, Deleted In Esophageal Cancer 1; EDD1, E3 ubiquitin-protein ligase; EDN1, Endothelin 1; EPO, Erythropoietin; FLK-1, Fetal liver kinase-1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GLUT1/3, Glucose transporter 1/3; GPI, glycosylphosphatidylinositol; GPX3, Glutathione peroxidase 3; HMOX1, heme oxygenase (decycling) 1; ID2, DNA-binding protein*

inhibitor; IGFBP3, Insulin-like growth factor-binding protein 3; ITF, Intestinal trefoil factor; L1CAM, L1 cell adhesion molecule; LEP, leptin; LDHA, Lactate dehydrogenase A; LOX, Lysyl oxidase; LOXL2/4, Lysyl oxidase homolog 2/4; MCL1, myeloid cell leukaemia sequence 1 protein; MMP-1/2/9/14, Matrix metalloproteinase 1/2/9/14; NDRG, N-myc downstream-regulated gene; NOXA, Phorbol-12-myristate-13-acetate-induced protein 1; NOS2/3, Nitric oxide synthase; NOX2, NADPH oxidase 2; NPM, Nucleophosmin/B23; PDGF-β, Platelet-derived growth factor-B; PDK1, Pyruvate dehydrogenase kinase 1; PFKL, 6-phosphofructokinase, liver type; PGF, Placental growth factor; PGK1, Phosphoglycerate kinase 1; PKM2, Pyruvate kinase M2; PP5, Serine threonine protein phosphatase type 5; SNAI1/2, Zinc finger protein; SOD2, Superoxide dismutase 2; STC2, Stanniocalcin 2; STK15, Aurora/Ipl-1 related serine/threonine kinase; TCF3, Transcription Factor 3; TGF2, Transforming growth factor 2; TGFβ3, Transforming growth factor-β3; TWIST1, Twist Family BHLH Transcription Factor 1; VEGF, Vascular Endothelial Growth Factor; VIM, Vimentin; ZEB1/2, Zinc finger E-box-binding homeobox 1/2 (Adapted from Iyer, Leung and Semenza, 1998; Liu *et al.*, 2012; Dengler, Galbraith and Espinosa, 2014).

4.1.2 HIF-1α and inflammation

Inflammation and tissue hypoxia have been correlated in a number of pathologies like inflammatory bowel disease (IBD), atherosclerosis, arthritis and obesity (Colgan and Taylor, 2010; Cummins *et al.*, 2016). Moreover, HIF-1α signalling has been described as a tumour-associated inflammatory indicator (Mantovani *et al.*, 2008; Mamlouk and Wielockx, 2013). Increased demand for oxygen in an inflammatory microenvironment (due to increase in metabolic activity), recruitment of inflammatory cells and disruption of oxygen delivery mechanisms are some of the circumstances favouring the activation of hypoxia signalling during prolonged and sustained inflammatory conditions (Cummins *et al.*, 2016). In that sense, immune cells like neutrophils have been shown to promote hypoxic conditions by consuming high amounts of oxygen in the oxidative burst (Campbell *et al.*, 2014; Campbell and Colgan, 2015). Like most cells, immune cells in a hypoxic microenvironment are subjected to activation of HIF-1α signalling (Cramer *et al.*, 2003; Walmsley *et al.*, 2005; Dang *et al.*, 2011; Scholz and Taylor, 2013). Myeloid cell as well as Th1 and Th17 cell functions have been described to be impaired in the absence of HIF-1α (Cramer *et al.*, 2003; Dang *et al.*, 2011; Higashiyama *et al.*, 2012). In contrast, HIF-1α seems to induce maturation of dendritic cells (DCs) in inflammatory conditions (Jantsch *et al.*, 2008). In the myeloid lineage (granulocytes, monocytes, macrophages), activation of HIF signalling promotes cell aggregation and motility, and is a mechanism for bacterial killing (Cramer *et al.*, 2003; Walmsley *et al.*, 2005; Scholz and Taylor, 2013). Studies in a mouse model of sepsis with conditional deletion of HIF-1α in the myeloid linage showed reduced lipopolysaccharide (LPS)-induced mortality and sepsis-associated symptoms (Peyssonnaux *et al.*, 2007). Moreover, HIF-1α favoured infiltration of inflammatory cells in a DSS-induced colitis model aggravating the disease by regulating the expression of macrophage migration inhibitory factor (MIF) (Shah *et al.*, 2009).

The changes seen in immune cells under hypoxic conditions have been associated with activation of the transcription factor nuclear factor-kappa B (NF-κB) (Bruning *et al.*, 2012). Considering NF-κB and HIF can both activate specific genes like interleukin-6 (IL-6), cyclooxygenase 2 (COX- 2), inducible nitric oxide synthase (NOS2), platelet endothelial cell adhesion molecule-1 (PECAM-1), matrix metalloproteinase 9 (MMP 9), BCL2, CXCR1 and CXCR2, it has been suggested they act together under hypoxic inflammation (Maxwell *et al.*, 2007; Bruning *et al.*, 2012; Hoesel and Schmid, 2013). The signal transducer and activator of transcription 3 (STAT3) has also been indicated to interact with HIF and NF-κB in the regulation of other genes involved in survival (like mcl-1), proliferation (like c-myc or cyclin D1), invasion (MMP 2) and angiogenesis (VEGF) (Jung *et al.*, 2005; Multhoff, Molls and Radons, 2012). Direct

interaction between STAT3 and HIF-1α has been shown to be crucial for activation of HIF target genes, and their association has been suggested to induce hypoxia-mediated immunoresistance in lung cancer cells (Noman *et al.*, 2009; Pawlus, Wang and Hu, 2014). A positive association with chemoresistance and tumour progression due to a sustained inflammatory milieu induced by interaction between HIF-1α and TLR4 has also been described in glioblastoma and pancreatic adenocarcinoma (Zhang *et al.*, 2010; Oblak and Jerala, 2011; Tewari *et al.*, 2012). TLR3 stimulation has also been shown to mediate metabolic reprogramming, and regulation of angiogenesis and apoptosis in a HIF-dependent mechanism in several types of cancer, including prostate cancer, head and neck carcinoma and pharyngeal cancer (Salaun *et al.*, 2006; Paone *et al.*, 2010; Veyrat *et al.*, 2016; Matijevic Glavan *et al.*, 2017). In addition, insulin resistance due to low-level inflammation triggered by the interaction between TRAF6, HIF-1α and IL1β has also been described in adipose tissue of a murine model of obesity (Yang *et al.*, 2014). Furthermore, transcription factors c-Jun and AP-1, important mediators of MAPKs in TLRs signalling, have also been shown to participate in regulation of gene expression under hypoxic conditions (Alfranca *et al.*, 2002; Salnikow *et al.*, 2002). Altogether these reports support a close interplay between innate immunity, inflammatory pathways and HIF signalling that will be further explored in this chapter.

4.2 Results

4.2.1 TLR pathways regulate levels of HIF-1α

Increasing evidence shows the interplay between the HIF pathway and inflammation. Hypoxic conditions have been linked to a number of inflammatory disorders like rheumatoid arthritis, inflammatory bowel disease and chronic infection (Scholz and Taylor, 2013). Furthermore, HIF signalling has also been shown to have a role in cancer and to mediate obesity related insulin-resistance and inflammation (Yang, Sharrocks and Whitmarsh, 2013; Lee *et al.*, 2014; Yang *et al.*, 2014; Balamurugan, 2016). Based on this knowledge, this study was keen to investigate the regulation of HIF by various inflammatory signalling pathways with particular focus on the pathways triggered by members of the TLR family. BMDMs were stimulated for 6 hours under normoxic conditions by Zymosan (TLR2 ligand), Poly(I:C) (TLR3 ligand), LPS (TLR4 ligand), Flagellin (TLR5 ligand), CL097 (TLR7/8 ligand) and CpG (TLR9 ligand) and the levels of HIF-1α were determined by immunoblot analysis. The expected band of 110kDa of full length HIF-1α was observed following the 6 hours treatment by all of the ligands listed above (**Figure 4.3A**). Whilst CpG induced stabilization of HIF-1α, it was the least effective (**Figure 4.3A**). Interestingly a number of the TLR ligands also promoted increased immunoreactivity with proteins migrating slightly faster than the 110kDa band and at 40kDa. Given that HIF stabilization is typically triggered by hypoxia, to mimic the hypoxic conditions, cells were incubated in a hypoxic chamber containing 1% O_2 for various time points and the levels of HIF-1α were measured by immunoblotting to assess if hypoxic conditions also promoted increased levels of proteins that migrate faster than 110kDa (**Figure 4.3B**). This resulted in increased levels of HIF-1α detected at 110kDa, but hypoxia failed to increase the levels of immunoreactive proteins of lower molecular weight. In order to further characterise the pattern of HIF-1α stabilisation in response to TLR ligands, the effects of Poly(I:C), a representative TLR ligand, were characterised in more detail by performing dose-dependent and kinetic studies. Dimethyloxaloylglycine (DMOG), well known for stabilizing HIF-1α by inhibiting PHD, was used as a positive control for stabilisation of HIF-1α under conditions that functionally mimic hypoxia. BMDMs were treated with DMOG for 8 hours or Poly(I:C) (25μg/mL) for 0-8 hours (**Figure 4.4A**). In an attempt to separate the faster migrating bands at 40kDa from the non-specific bands detectable in all of the samples, the SDS-PAGE gels were electrophoresed for longer time periods. Once again, Poly(I:C) was seen to promote the HIF-1α stabilisation migrating at 110kDa. Interestingly the faster migrating band at 40kDa was also detected following 4 hour and up to 8 hours stimulation.

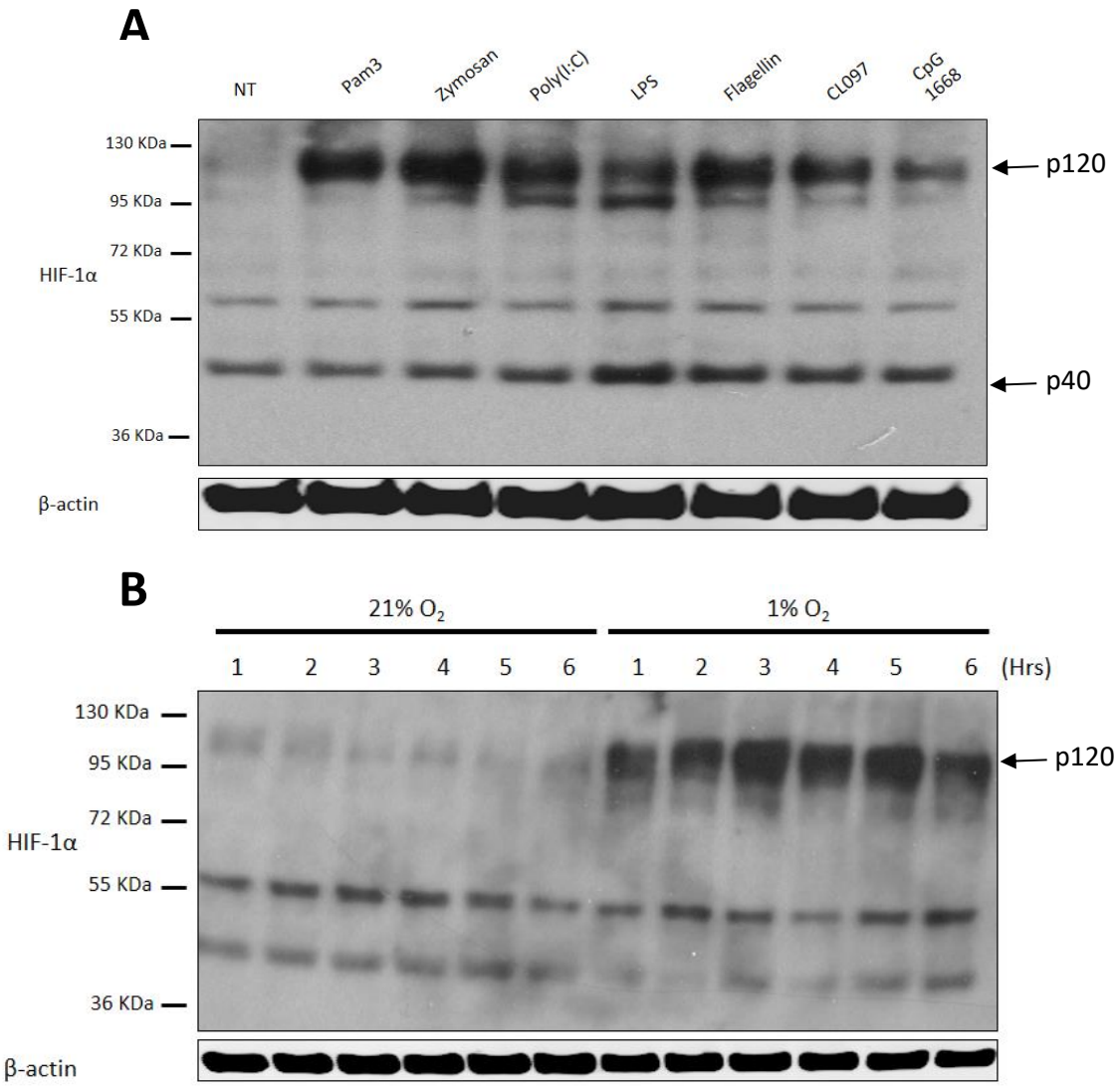

Figure 4.3 Effect of TLR stimulation and hypoxia on levels of HIF-1α

Immunoblot analysis of HIF-1α (120 kDa) in cell lysates from WT BMDMs stimulated with **(A)** 1µg/mL Pam3 (TLR1/2), 1µg/mL Zymosan (TLR2), 25µg/mL Poly(I:C) (TLR3; High molecular weight – HMW), 100ng/mL LPS (TLR4), 1µg/mL Flagellin (TLR5), 1µg/mL CL097 (TLR7/8) and 2µg/mL CpG 1668 (TLR9) for 6 hours. **(B)** Immunoblot analysis of HIF-1α (120 kDa) in cell lysates from WT BMDMs under normoxia (21% O_2) and hypoxia (1% O_2) conditions. β-actin (42 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

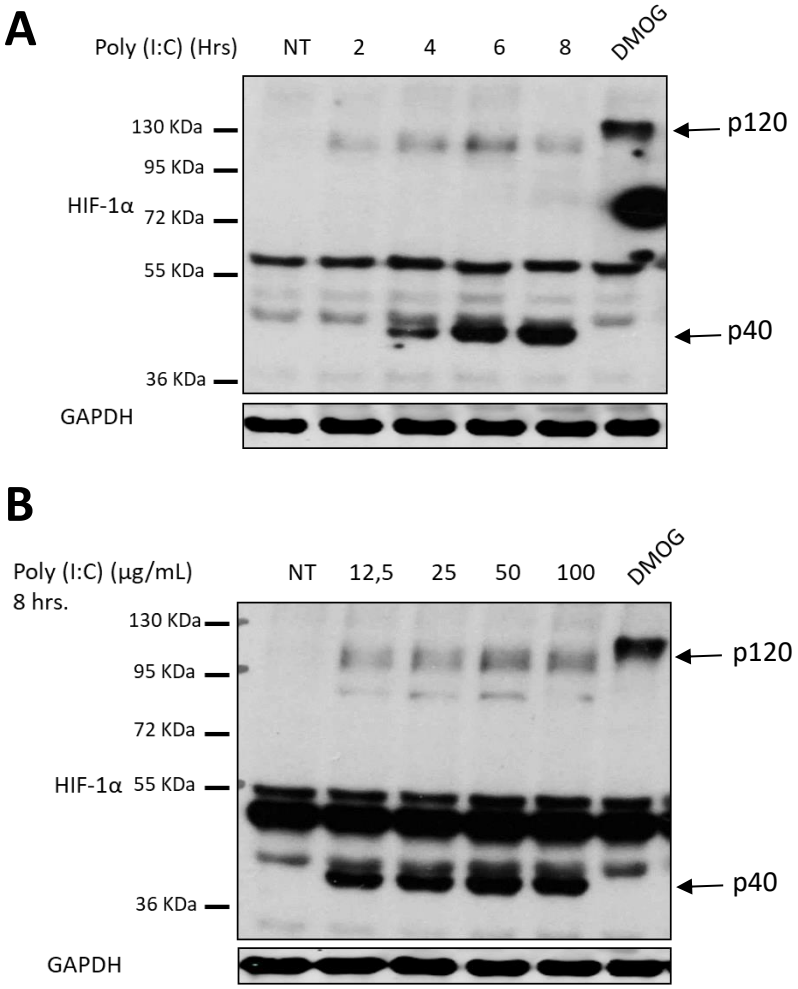

Figure 4.4 Dose-dependent and kinetic analysis of the effect of Poly(I:C) on HIF-1α.

Immunoblot analysis of HIF-1α (120 kDa) in cell lysates from WT BMDMs stimulated with **(A)** 25 µg/mL for 2, 4, 6 and 8 hrs, or **(B)** increasing concentrations of Poly(I:C) (12,5 - 100 µg/mL) for 8 hours. DMOG (0.5 mM) was used as control for stabilization of HIF-1α. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

HIF-1α stabilisation was also assessed following 8 hours stimulation with DMOG or increased concentrations of Poly (I:C) (12.5 – 100μg/mL). Both, the faster (40kDa) and the slower (110kDa) migrating bands were detected following the stimulations with Poly (I:C). However, stimulation of cells with DMOG resulted in expression of the higher molecular weight form only of HIF-1α (**Figure 4.4B**). Interestingly the latter migrates slower than the 110kDa band stabilised by Poly(I:C). These data suggest that whilst TLRs, like hypoxia, can promote increased levels of HIF-1α, they differ from hypoxia in relation to the electrophoretic mobilities of the proteins that are detected by the anti-HIF-1α antibody. The faster migrating form that manifests in response to TLR stimulation may represent a processed form of HIF-1α that is not apparent in response to hypoxia.

Studies next addressed if the lower molecular weight form of HIF-1α could represent a TLR-induced processing event, and if processing was facilitated by either of the 2 main protein degradation pathways in cells namely the ubiquitin-proteasome system or lysosomal-mediated processing. In order to examine if TLR signalling could trigger processing of full length HIF-1α (110kDa) to the smaller 40kDa form, full length HIF-1α was first stabilised with the PHD (prolyl hydroxylase domain-containing proteins) inhibitor Dimethyloxaloylglycine (DMOG), followed by LPS or Poly (I:C) treatment HIF-1α. DMOG interferes with PHD-dependant hydroxylation of HIF-1α thus precluding ubiquitination and degradation resulting in HIF-1α up-regulation (Baader *et al.*, 1994). As expected the upper band of HIF-1α was strongly induced with DMOG alone but notably the low molecular weight form was not detectable (**Figure 4.5**). Stimulation of the cells with the TLR3 and TLR4 ligands, Poly(I:C) and LPS respectively, resulted in increased levels of the higher and lower molecular weight forms of HIF-1α but interestingly neither ligand promoted augmentation of the levels of the lower form of HIF-1α when co-stimulated with DMOG. Phosphorylation of TBK1 and p65 was also examined to confirm signalling downstream of TLR3 and TLR4. This suggests that TLR signalling cannot directly process full length HIF-1α already stabilised by DMOG.

Next, we examined the ability of the lysosome to process HIF-1α into the faster migrating form of 40kDa following TLR activation. Chloroquine, a lysosomotropic agent was found to block the ability of Poly(I:C) to stabilise both the 110 and 40kDa forms of HIF-1α whereas, it was without effect on the ability of LPS to stabilise both forms (**Figure 4.5**). Furthermore, Chloroquine blocked TLR3-induced phosphorylation of TBK1 and p65 but did not affect their activation by TLR4. The lack of effect of Chloroquine on the levels of the 40kDa protein induced by LPS suggests that the lysosome does not mediate any processing of full length 110kDa HIF-1α to this lower molecular weight form. However, Chloroquine was very

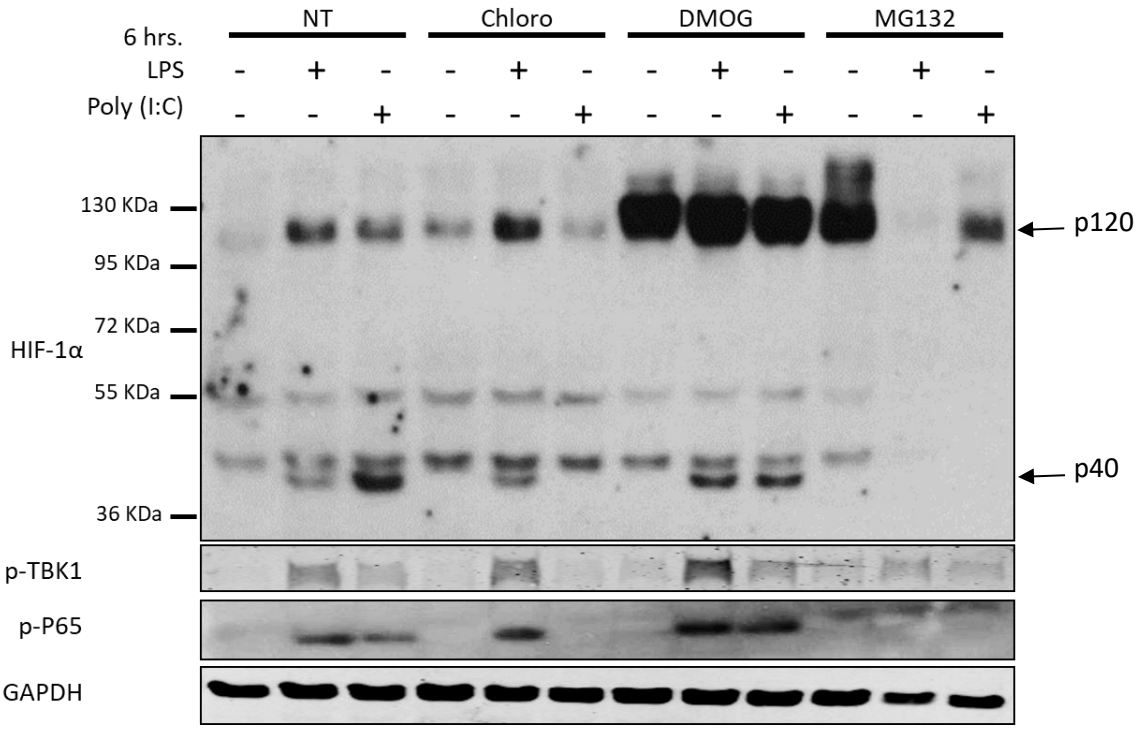

Figure 4.5 Effect of Chloroquine, MG132 and DMOG on levels of faster migrating form of HIF-1α in BMDMs.

Immunoblot analysis of HIF-1α (120 kDa), phosphorylated (p-) TBK1 (84 kDa) and p-P65 (65 kDa) in cell lysates from WT BMDMs pre-treated for 1 hour with 20 µM Chloroquine, 20 µM MG132 or 0,5mM DMOG, followed by 6 hours treatment with 25 µg/mL Poly(I:C) or 40 ng/mL LPS. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of four experiments.

effective in blocking the responses of the TLR3 ligand Poly(I:C). It should be noted that the lysosomotropic agent Chloroquine interferes with endosomal acidification (Steinman *et al.*, 1983). TLR3 is predominantly located in endosomes and TLR4 is endocytosed into endosomes to initiate TRAM-TRIF signalling (Akira and Takeda, 2004; O'Neill, Golenbock and Bowie, 2013). Furthermore, endosomal acidification is essential for an adequate activation of TLR3 (Pohar *et al.*, 2014). The selective inhibitory effects of Chloroquine on the response to Poly(I:C) and not LPS suggests that endosomal TLR3 can trigger downstream signalling to upregulate both forms of HIF-1α whereas plasma membrane located TLR4 is sufficient to achieve the same response.

Given that the lysosome appears to play a selective role in early TLR3 activation and not any processing of HIF-1α, studies were next performed using the proteasome inhibitor MG132 to examine if the proteasome contributes to possible processing of full length HIF-1α to the smaller 40kDA form. MG132 acts as an inhibitor of the 20S proteasome and calpain (Tsubuki *et al.*, 1996). As HIF-1α is mainly degraded by the proteasome, after hydroxylation by PHDs and ubiquitination by pVHL, MG132 induces strong accumulation of the 110kDa form of HIF-1α (**Figure 4.5**). Cells were also treated with LPS or Poly(I:C) in order to assess whether these ligands could still induce the formation of the smaller 40kDa form in the presence of MG132. However, there was a dramatic reduction in the levels of 110 and 40kDa forms of HIF when cells were co-treated with Poly(I:C) and MG132 with total loss of both forms when the latter was co-treated with LPS (**Figure 4.5**). Phosphorylation p65 was also undetectable when the cells were treated with MG132 followed by LPS or Poly(I:C) while phosphorylation of TBK was reduced but remained evident. These results were expected as phosphorylation of p65 depends on proteasomal degradation of NF-κB inhibitory protein IκB, but activation of TBK it is independent of the proteasome pathway.

Since co-stimulation with MG132 and TLR ligands appear to block all downstream signalling, we were keen to assess if this was due to cytotoxicity. We were especially interested in exploring whether the cells were undergoing apoptosis since, as described in the previous chapter, NF-κB is a pro-survival factor by upregulating anti-apoptotic proteins such as c-FLIP, IAPs and Bcl proteins, and MG132 will inhibit NF-κB activation by blocking proteasomal degradation of the NF-κB inhibitory protein IκB. BMDMs were pre-treated with MG132 for 1 hour followed by LPS stimulation for 30 minutes, 1 hour, 3 hours or 6 hours (**Figure 4.6A**). As expected the expression of HIF-1α stabilises following 3 hours and 6 hours treatment of MG132 alone. Stimulation with LPS alone for 6 hours also induced HIF-1α stabilization, as did 1-hour pre-treatment with MG132 followed by 3 hours stimulation with the TLR4 ligand, LPS. However,

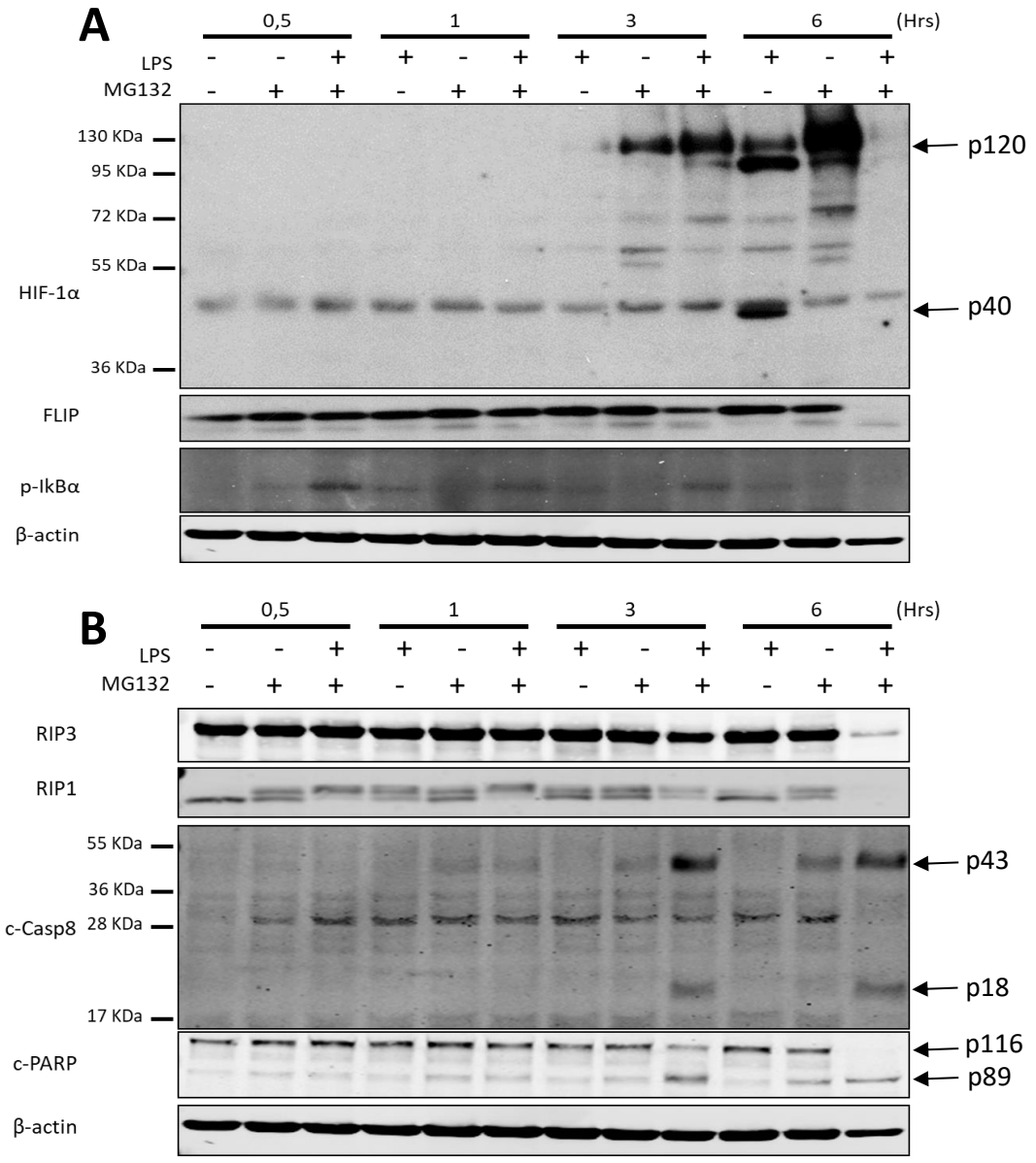

Figure 4.6 Proteasome inhibition and TLR4 stimulation induce apoptosis in BMDMs.

Immunoblot analysis of **(A)** HIF-1α (120 kDa), FLIP (55 kDa) and phosphorylated (p-) IκBα (40 kDa), or **(B)** p-MLKL (54 kDa), RIP3 (46-53 kDa), RIP1 (74 kDa), cleaved (c-) Caspase 8 (18 and 43 kDa) and c-PARP (89 and 116 kDa) in cell lysates from WT BMDMs pre-treated for 1 hour with 20 µM MG132, followed by 30 minutes, 1, 3 or 6 hours stimulation with 40 ng/mL LPS. β-actin (42 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

as previously seen, the 110kDa HIF-1α band was undetectable when co-stimulated with MG132 (1-hour pre-treatment) and LPS (6 hours treatment). Cytotoxicity was then evaluated by determining the expression of apoptotic molecular markers such as cleaved Caspase 8 and PARP (**Figure 4.6B**). As described in section 3.1.1.1, cleavage of caspase proteins is essential for their activation resulting in formation of two subunits p43/p41 and p18 following Caspase 8 activation, and p17 and p19 following caspase 3 activation. In addition, the processing of DNA-repair protein Poly (ADP-ribose) polymerase (PARP) from its full length p116 into its cleaved form p89 was also examined as one of the apoptotic markers. Cleavage of both caspase 8 and PARP was induced following 1-hour pre-treatment with MG132 and 3 hours or 6 hours LPS stimulation. The inhibitor alone appeared to induce the cleavage of caspase 8 and PARP at the same time points but to a lesser extent than when combined with LPS. Next, the expression of RIP kinases, RIP3 and RIP1 was evaluated, considering their essential role in mediating different types of cell death regulation (**Figure 4.6B**). Both RIP3 and RIP1 were consistently expressed following 30 minutes and up to 6 hours stimulation, with no upward shift of RIP3 observed at any time point, as would be expected if necroptosis signalling was activated. Interestingly, a double band was seen for RIP1 when treated with MG132 or LPS, or the combination of both at different time points. Such observation is most likely reflecting post-translational modification of RIP1, such as phosphorylation or ubiquitination, induced by the treatments. Furthermore, following co-treatment with MG132 (1-hour pre-treatment) and 3 hours LPS treatment, the expression of RIP3 and RIP1 was decreased and completely diminished when LPS was left for 6 hours. Evidently, inhibition of the proteasome with MG132 blocks NF-κB activation and the additional stimulation with TLR4 ligand LPS drives the signalling pathway towards apoptosis, explaining the disappearance of the higher molecular weight form of HIF-1α.

Studies next examined if the processing of HIF-1α following activation of TLR3 and TLR4 was cell specific. In order to address this, murine embryonic fibroblast (MEFs) were pre-treated for 1 hour with either Chloroquine, DMOG or MG132 followed by stimulation with LPS or Poly(I:C) for 6 hours as previously done in BMDMs (**Figure 4.7**). Some stabilization of the higher molecular weight band of HIF-1α was seen following treatment with ligands alone but more interestingly, the lower migrating band of HIF-1α is undetectable following stimulation with Poly(I:C) and LPS in this cell type suggesting cell specificity. It is to note that the HIF-1α upper band stabilization was considerably stronger with DMOG and MG132 as expected, due to their role in interfering with HIF-1α proteasomal degradation. However, there was no decrease or loss of this upper band with the combination of MG132 and either LPS or Poly(I:C) as seen in macrophages. In contrast, the use of Chloroquine had negligible effect on the expression of the

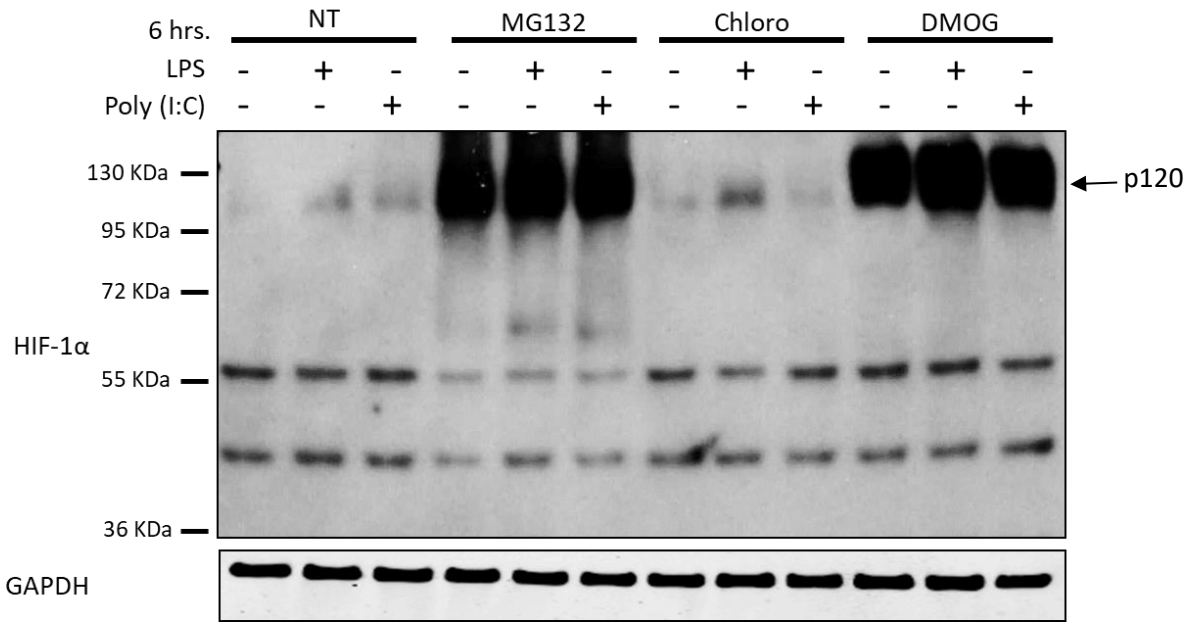

Figure 4.7 Effect of Chloroquine, MG132 and DMOG on levels of faster migrating form of HIF-1α in MEFs.

Immunoblot analysis of HIF-1α (120 kDa) in cell lysates from WT MEFs pre-treated for 1 hour with 20 µM Chloroquine, 20 µM MG132 or 0,5mM DMOG, followed by 6 hours treatment with 25 µg/mL Poly(I:C) or 40 ng/mL LPS. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

upper band, as the pattern was similar to stimulation with LPS or Poly(I:C) only. All together these results suggest the TLR3/4-induced lower band of HIF-1α is specific to macrophages, as well as the activation of apoptosis induced by proteasome inhibition and TLR4 stimulation with LPS.

The possibility that the appearance of the lower migrating HIF-1α band induced by TLR3/4 activation could affect the expression of cytokines (TNFα, IL6 or IL10) (**Figures 4.8 and 4.10**) and HIF responsive genes (**Figures 4.9 and 4.11**) was next examined. To evaluate cytokine production, BMDMs were treated with the corresponding inhibitors for 1 hour followed by stimulation with LPS or Poly(I:C) for 24 hours to allow for accumulation of the cytokines in the supernatant (**Figures 4.8 and 4.10**). As expected, activation of TLR4 by LPS alone resulted in induction of TNFα, IL6 and IL10 (**Figure 4.8**). However, when the cells were co-stimulated with DMOG (1-hour pre-treatment) and LPS a reduction in IL6 and IL10 production was seen. In turn, Chloroquine stimulation did not alter cytokine expression when compared with LPS treatment alone. These results correlate with previous observations where neither of the inhibitors were found to exert any change to the lower migrating band of HIF-1α. In contrast, inhibition of the proteasome with MG132 alone moderately increased the expression of TNFα, and strongly induced the expression of IL10. The latter effect prompted further questions regarding the capacity of the proteasomal inhibitors to induce this anti-inflammatory cytokine and this formed the focus of Chapter 5 of this thesis. Furthermore, the combined treatment of MG132 and LPS significantly reduced expression of TNFα, IL6 and IL10. Considering our previous findings showing triggering of apoptotic mechanism under proteasome inhibition and TLR4 stimulation, the reduction of cytokines under these conditions could be explained as a secondary effect of such program cell death pathway.

The effect on HIF responsive genes was also assessed using BMDMs pre-treated with the above listed inhibitors followed by 6 hours stimulation with LPS. Messenger RNA (mRNA) was extracted and the levels of CXCL10, glucose transporter GLUT-1 and vascular endothelial growth factor (VEGF) were analysed by RT-PCR (**Figure 4.9**). CXCL10 was evaluated as a positive control of antimicrobial response induced by LPS, while GLUT-1 and VEGF are known to be induced under hypoxic conditions in a HIF-1α-dependant signalling (Sánchez-Elsner *et al.*, 2001; Hayashi *et al.*, 2005; Liu *et al.*, 2012). Stimulation of the cells with TLR4 ligand LPS increased CXCL10 mRNA levels but did not change GLUT-1 and VEGF mRNA levels. This observation was expected considering TLR4 activation induces strong pro-inflammatory activation with chemokines like CXCL10 while GLUT-1 and VEGF are HIF specific responsive genes. In addition, the time point evaluated was not long enough to induce the expression of GLUT-1 and VEGF in

a TLR4 signalling-dependant activation. In contrast, the mRNA levels of both HIF responsive genes were seen to increase considerably with DMOG. Such effect was predictable given this inhibitor mediates HIF stabilization by blocking its hydroxylation. The combined treatment of DMOG and LPS had a moderate effect on GLUT-1 and VEGF mRNA levels with tendency to reduction. Meanwhile, Chloroquine had a negligible effect on CXCL10, GLUT-1 or VEGF mRNA levels by itself or when combined with LPS. Finally, proteasome inhibitor MG132 treatment alone increased GLUT-1 and VEGF levels modestly, with further decrease of GLUT-1 when combined with LPS stimulation. The levels of mRNA for the genes evaluated do not seem to reflect changes that could be correlated to TLR4-induced lower band of HIF-1α.

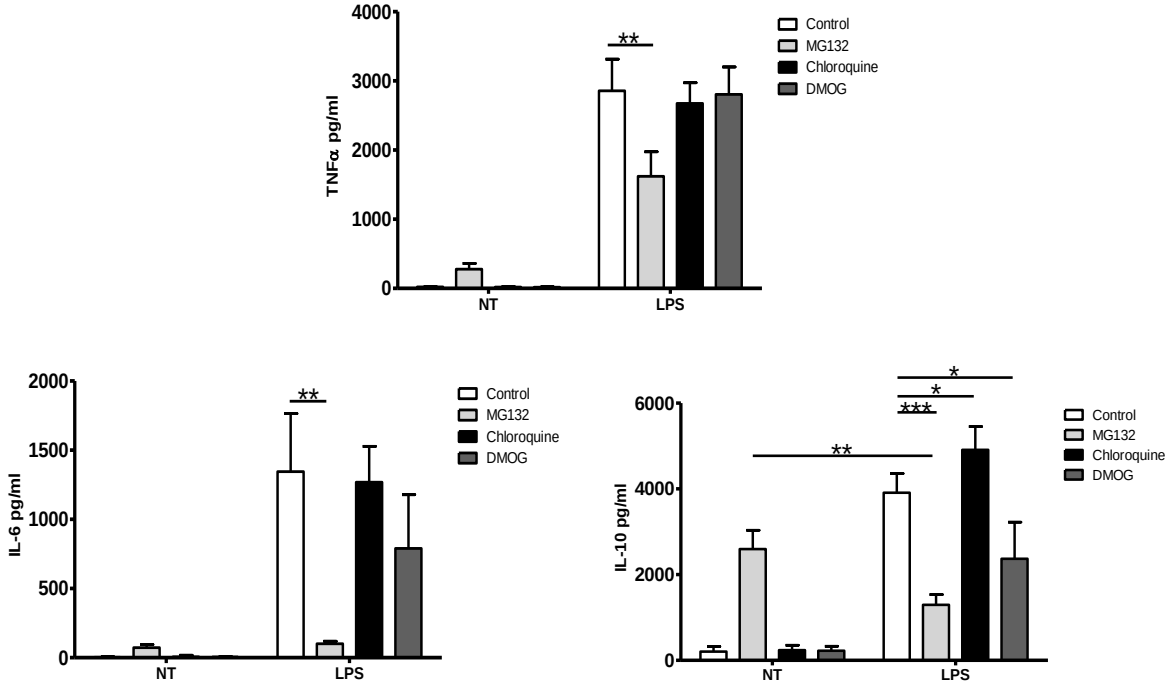

Figure 4.8 Effect of Chloroquine, MG132 and DMOG on cytokine expression in BMDMs treated with LPS.

ELISA analysis of cytokines TNFα (upper panel), IL6 (left-lower panel) and IL10 (right-lower panel) from supernatants of WT BMDMs pre-treated for 1 hour with 20 µM Chloroquine, 20 µM MG132 or 0,5mM DMOG, followed by 24 hours stimulation with 40 ng/mL LPS. Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

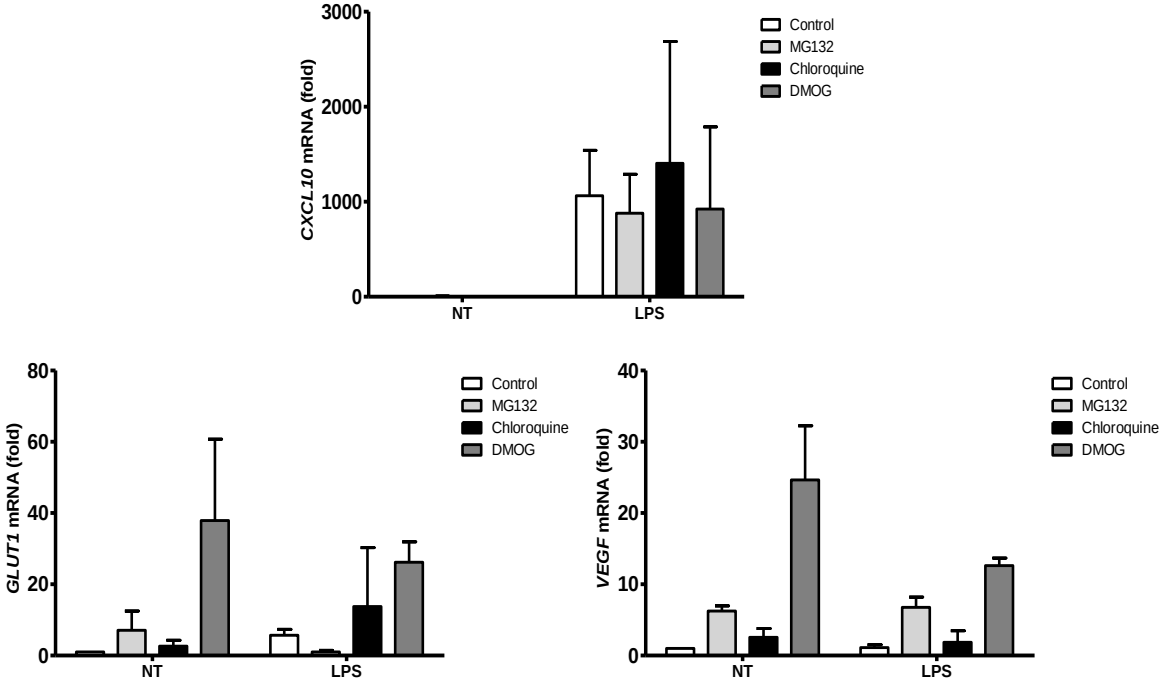

Figure 4.9 Effect of Chloroquine, MG132 and DMOG on genes activated by HIF-1α signalling in BMDMs treated with LPS.

Quantitative PCR of mRNA expression for CXCL10 (upper panel), GLUT1 (left-lower panel) and VEGF (right-lower panel) in cell lysates of WT BMDMs pre-treated for 1 hour with 20 µM Chloroquine, 20 µM MG132 or 0,5mM DMOG, followed by 6 hours stimulation with 40 ng/mL LPS. Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. No statistical significance was found.

Similar analysis on cytokine expression and mRNA levels were performed using Poly(I:C) to determine possible variations related to the TLR3-induced lower migrating band (**Figures 4.10 and 4.11**). Poly(I:C) stimulation also increased the expression of all the cytokines (TNFα, IL6 or IL10), however, the expression was not as strong as that observed with LPS treatment (**Figure 4.10**). Cytokine expression was unaffected following the treatment with inhibitors, DMOG and Chloroquine alone, while MG132 increased IL10 (this will be further addressed in Chapter 5). However, the co-treatment of either DMOG or MG132 with Poly(I:C) significantly decreased the expression of TNFα, IL6 and IL10. In the case of Chloroquine with Poly(I:C), a reduction in the cytokines was expected as this TLR3 ligand requires acidification to bind the receptor, and acidification is blocked by Chloroquine. Meanwhile, the reduction in cytokine production following stimulation with DMOG or MG132, which promote HIF stabilisation, and Poly(I:C) could indicate cross-regulation between TLR3 and HIF signalling. In addition to cytokine expression, the effects on CXCL10, GLUT-1 and VEGF mRNA levels were also explored. BMDMs were pre-treated with DMOG, MG132 or Chloroquine followed by Poly(I:C) treatment (**Figure 4.11**). TLR3-ligand by itself induced poor expression of GLUT-1 and VEGF, but increased CXCL10 levels. DMOG, as seen with LPS, induced GLUT-1 and VEGF by itself and both were reduced when co-treated with Poly(I:C), where VEGF mRNA levels were found to be significantly lower when co-treated with DMOG and Poly(I:C) compared to DMOG treatment alone. Chloroquine had no major effect on CXCL10, GLUT-1 and VEGF mRNA levels, except for a tendency to reduce CXCL10 levels when co-treated with Poly(I:C). Finally, with the proteasome inhibitor MG132, there was a reduction in GLUT-1 but not VEGF levels when co-stimulated with Poly(I:C), similar to results seen with MG132 and LPS co-stimulation.

Considering TLRs can trigger metabolic re-programming from oxidative phosphorylation to glycolysis in innate immune signalling to trigger inflammation, studies next addressed the effect of blocking glycolysis on TLR-regulation of HIF-1α (**Figures 4.12 to 4.14**). A modified glucose molecule known as 2-Deoxy-D-glucose (2-DOG) was used as inhibitor of glycolysis. 2-DOG acts a competitive inhibitor by blocking production of glucose-6-phosphate from glucose (Wick *et al.*, 1957). BMDMs were treated with the inhibitor for 1 hour followed by either Poly(I:C) or LPS stimulation for 6 hours. Treatment with 2-DOG reduced TLR3/4-induced upper and lower band of HIF-1α when compared with treatment with ligands only (**Figure 4.12**). These findings suggest that glycolysis, one of the central processes regulated by HIF signalling, can positively regulate HIF expression. The effects of glycolysis inhibition and TLR3/4 activation on cytokine expression (**Figures 4.13A and 4.14A**) and HIF responsive genes (**Figures 4.13B and 4.14B**) were

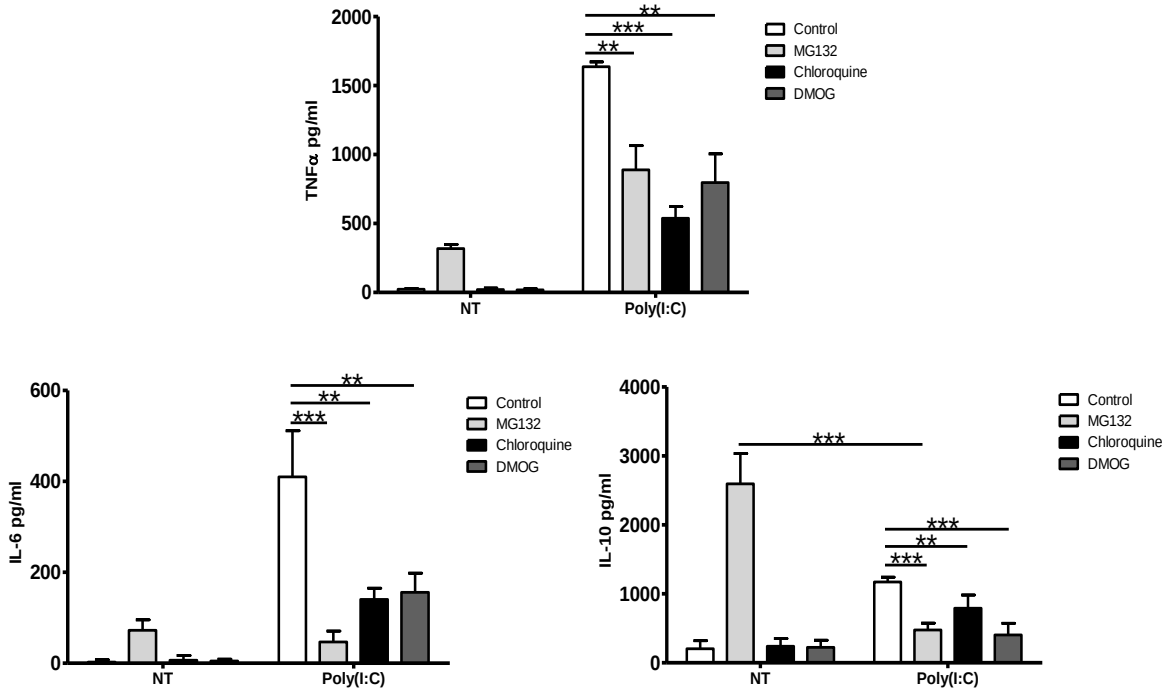

Figure 4.10 Effect of Chloroquine, MG132 and DMOG on cytokine expression in BMDMs treated with Poly(I:C).

ELISA analysis of cytokines TNFα (upper panel), IL6 (left-lower panel) and IL10 (right-lower panel) from supernatants of WT BMDMs pre-treated for 1 hour with 20 µM Chloroquine, 20 µM MG132 or 0,5mM DMOG, followed by 24 hours stimulation with 25 µg/mL Poly(I:C). Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001.

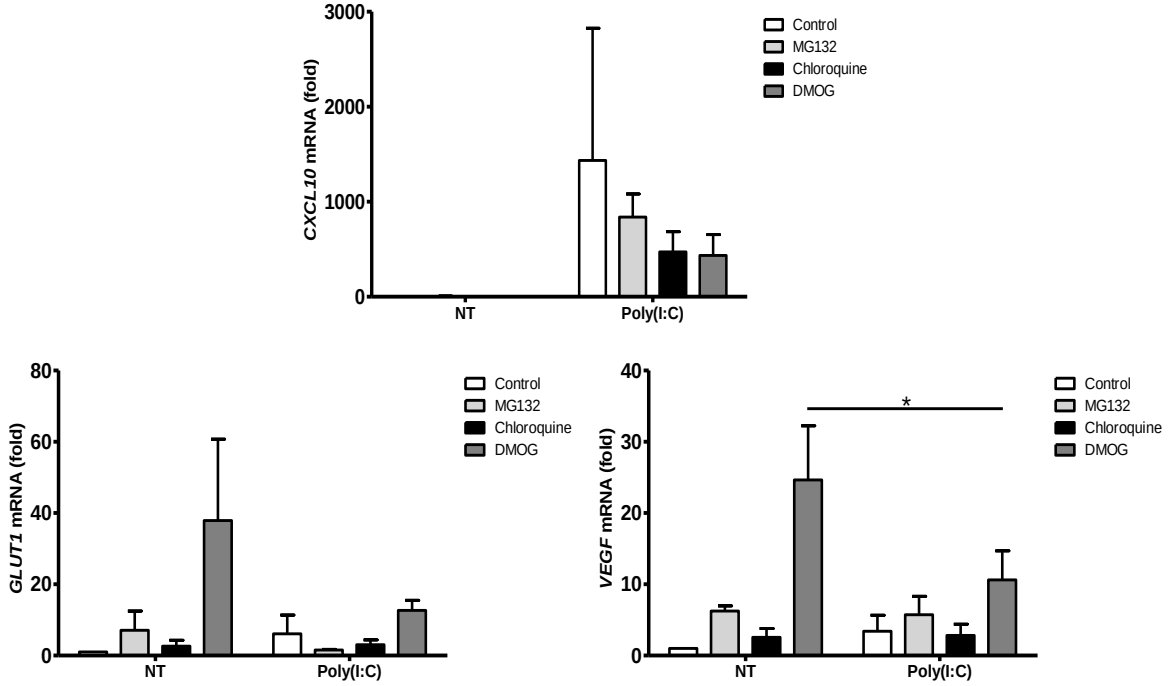

Figure 4.11 Effect of Chloroquine, MG132 or DMOG on HIF-1α-responsive genes in BMDMs treated with Poly(I:C).

Quantitative PCR of mRNA expression for CXCL10 (upper panel), GLUT1 (left-lower panel) and VEGF (right-lower panel) in cell lysates of WT BMDMs pre-treated for 1 hour with 20 µM Chloroquine, 20 µM MG132 or 0,5mM DMOG, followed by 6 hours stimulation with 25 µg/mL Poly(I:C). Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. *$p < 0.05$.

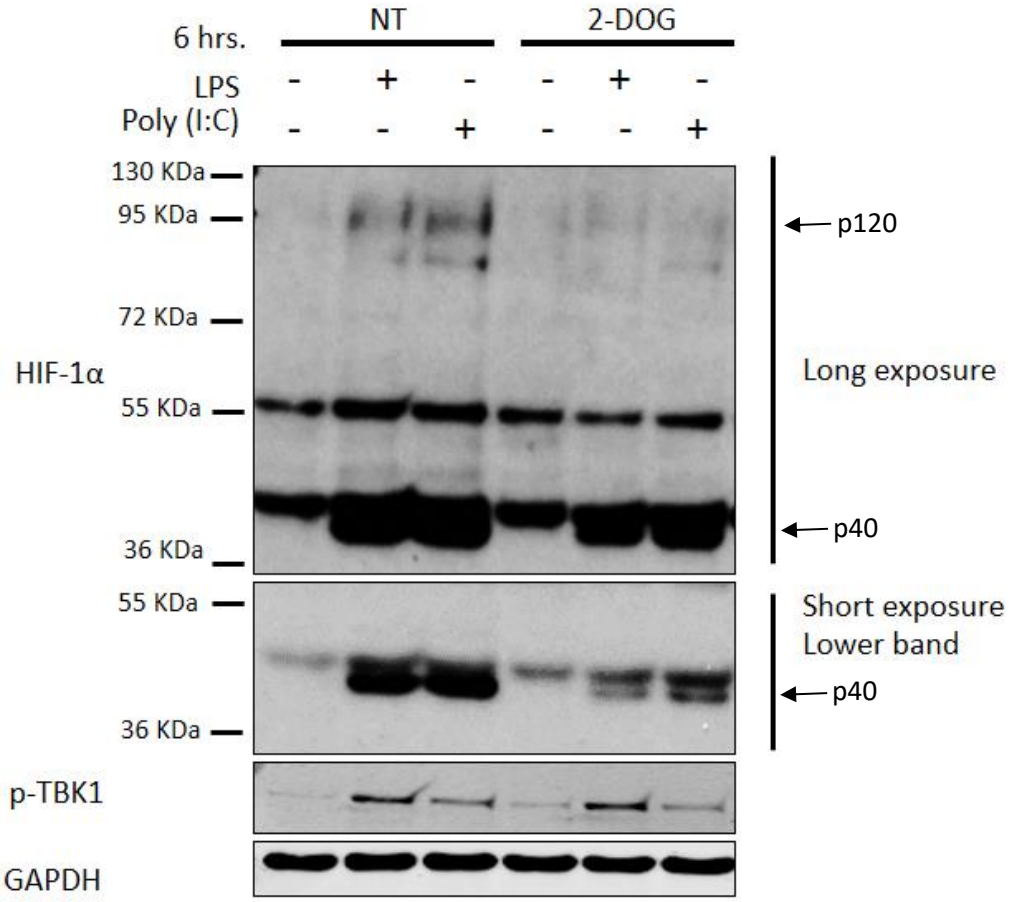

Figure 4.12 Effect of 2-DOG on HIF-1α in BMDMs.

Immunoblot analysis of HIF-1α (120 kDa) and phosphorylated (p-) TBK1 (84 kDa) in cell lysates from WT BMDMs pre-treated for 1 hour with 5 mM 2-DOG, followed by 6 hours stimulation with 25 µg/mL Poly(I:C) or 40 ng/mL LPS. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Long exposure corresponds to up to one hour or longer time of exposure of the film to the membranes cover with ECL, and short exposure to less than half an hour exposure of the film. Data are representative of three experiments.

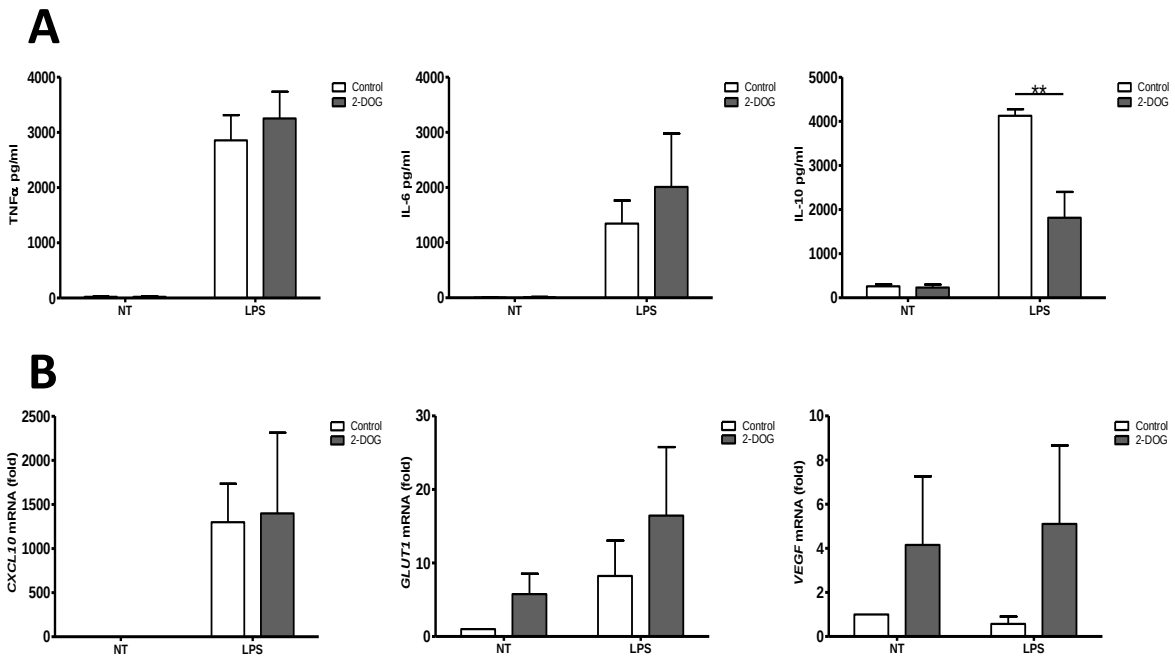

Figure 4.13 Effect of 2-DOG on expression of cytokines and HIF-1α-responsive genes in BMDMs treated with LPS.

(A) ELISA analysis of cytokines TNFα (left panel), IL6 (centre panel) and IL10 (right panel) in supernatant and **(B)** quantitative PCR of mRNA expression for CXCL10 (left panel), GLUT1 (centre panel) and VEGF (right panel) in cell lysates of WT BMDMs pre-treated for 1 hour with 5 mM 2-DOG, followed by 24 hours stimulation with 40 ng/mL LPS. Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001.

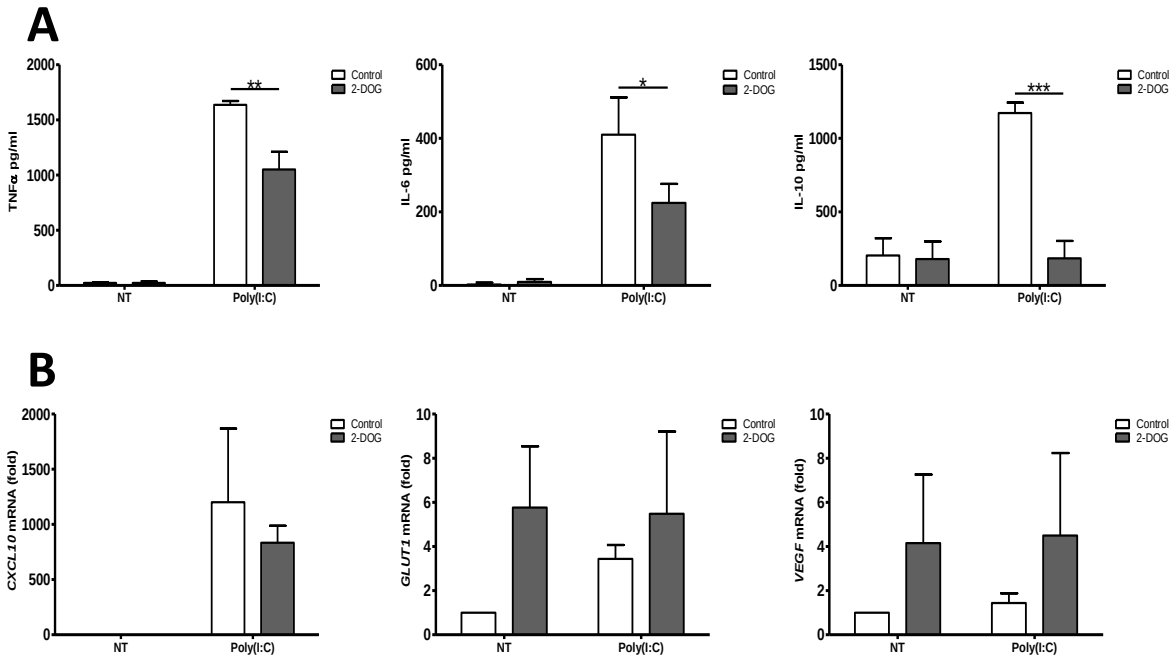

Figure 4.14 Effect of 2-DOG on cytokine expression and HIF-1α-responsive genes in BMDMs treated with Poly(I:C).

(A) ELISA analysis of cytokines TNFα (left panel), IL6 (centre panel) and IL10 (right panel) in supernatant and **(B)** quantitative PCR of mRNA expression for CXCL10 (left panel), GLUT1 (centre panel) and VEGF (right panel) in cell lysates of WT BMDMs pre-treated for 1 hour with 5 mM 2-DOG, followed by 24 hour stimulation with 25 μg/mL Poly(I:C). Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

next examined. Blocking glycolysis did not increase the expression of TNFα, IL6 and IL10 alone but was seen to reduce IL10 expression induced by TLR4 ligand LPS (**Figure 4.13A**). Furthermore, 2-DOG treatment alone augmented GLUT-1 and VEGF mRNA levels (**Figure 4.13B**). In contrast to TLR4 signalling, blocking glycolysis followed by TLR3 stimulation with Poly(I:C) significantly reduced the expression of TNFα, IL6 and IL10 (**Figure 4.14A**). However, the combined treatment of 2-DOG and Poly(I:C) did not change GLUT-1 and VEGF mRNA levels compared to 2-DOG alone (**Figure 4.14B**). These results show glycolysis can regulate HIF upper and lower bands and expression of cytokines but does not affect GLUT-1 and VEGF mRNA levels.

Altogether these findings point at a close interplay between HIF and TLR3 signalling. The immunoblotting analysis suggest the lower migrating form of HIF-1α might be induced in a TRIF-dependant signalling. It also clearly indicates that such effect is restricted to macrophages and can affect the expression of pro and anti-inflammatory cytokines activated by TLR3/4 signalling. In addition, metabolic re-programming, that is triggered by TLRs, appears to play an important role in regulation of HIF-1α.

4.2.2 Investigating TLR3/4 downstream signalling on induction of faster migrating form of HIF-1α

Whilst the pathways mediating hypoxia-induced stabilisation of HIF-1α are well understood the early signalling mechanisms that mediate the regulation of HIF-1α by TLRs are not well characterised. To this end, studies were then performed to map the receptor proximal signalling pathways that are likely to be employed by TLRs to drive increased levels of HIF-1α. We initially explored the role of signalling downstream of TRIF in mediating the response to Poly (I:C) and LPS (**Figure 4.15**). WT and TRIF- or IRF3- deficient immortalized BMDMs (iBMDMs) were stimulated with LPS or Poly(I:C) for 4 or 6 hours. The higher migrating band of HIF-1α was strongly induced in WT iBMDMs following stimulation with both TLR-ligands for 4 and 6 hours (**Figure 4.15**). Meanwhile, the lower migrating band of HIF-1α was apparent at 6 hours treatment with both LPS and Poly(I:C) but, contrary to the observation in primary cells, it was less prominently induced by Poly(I:C). The expression of the higher migrating band of HIF-1α in *trif*$^{-/-}$ iBMDMs was similar to WT control cells. However, the lower migrating band disappeared in the cells deficient for TRIF compared with WT cells, potentially indicating a crucial role of TRIF signalling in inducing the lower form of HIF-1α (**Figure 4.15A**). The next step was to probe signalling downstream of TRIF and the role of IRF3 was evaluated using iBMDMs deficient for this transcription factor (**Figure 4.15B**). IRF3-deficient cells showed reduced expression of the higher migrating form of HIF-1α for both treatments with LPS and Poly(I:C). Moreover, the expression of HIF-1α lower migrating band was also clearly reduced in *irf3*$^{-/-}$ iBMDMs treated with LPS and completely lost when the cells were treated with Poly(I:C) (**Figure 4.15B**). These results indicate the TLR3-induced lower migrating form of HIF-1α depends on IRF3 activation, while TLR4-induced lower migrating band may require additional signalling pathways such as the MyD88 pathway that is mediated by interleukin-1 receptor-associated kinase (IRAKs).

The role of early molecular signalling downstream of TLR4 specifically mediated by IRAKs was next explored. Primary WT BMDMs were treated with LPS or Poly(I:C) in the presence or absence of a benzimidazole compound which selectively inhibitors IRAK1 and IRAK4. Cells were pre-treated with the inhibitor for 1 hour and then stimulated with the ligands for TLR4 and TLR3 for 6 hours (**Figure 4.16**). Expression of HIF-1α upper and lower bands was apparent in cells subjected to either LPS or Poly(I:C) treatment. Inhibition of IRAK1/4 did not affect the expression of the higher migrating form of HIF-1α however, the expression of lower migrating form of HIF-1α in response to LPS, was diminished when the cells were treated with the inhibitor and it modestly reduced the levels that were manifested in response to Poly(I:C). All together, these

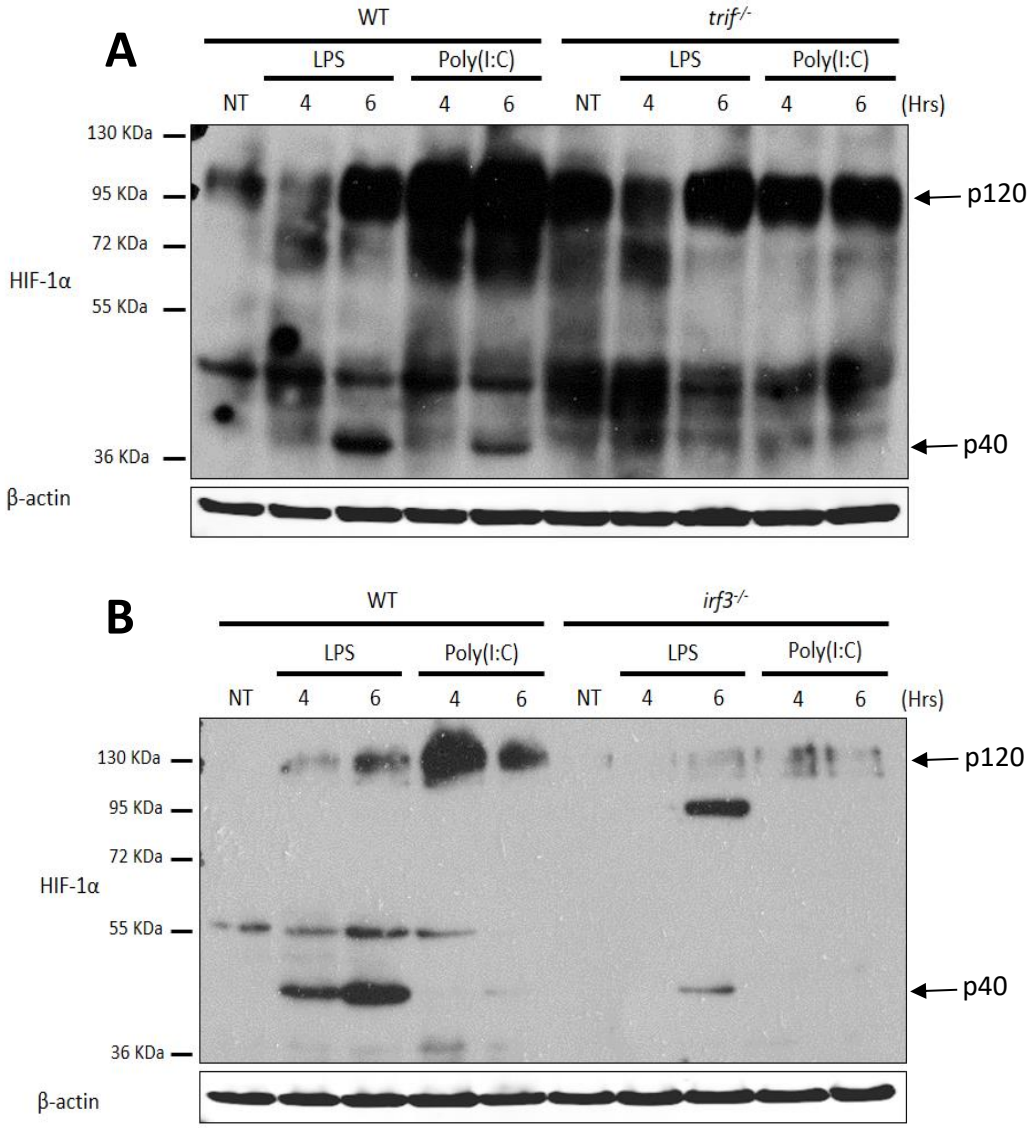

Figure 4.15 TLR4/3-induced stabilisation of HIF-1α is dependent on TRIF and IRF3 signalling.

Immunoblot analysis of HIF-1α (120 kDa) in cell lysates from **(A)** WT and *TRIF*$^{-/-}$, or **(B)** WT and *IRF3*$^{-/-}$ immortalized BMDMs stimulated for 4 or 6 hours with 40 ng/mL LPS or 25 μg/mL Poly(I:C). β-actin (42 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

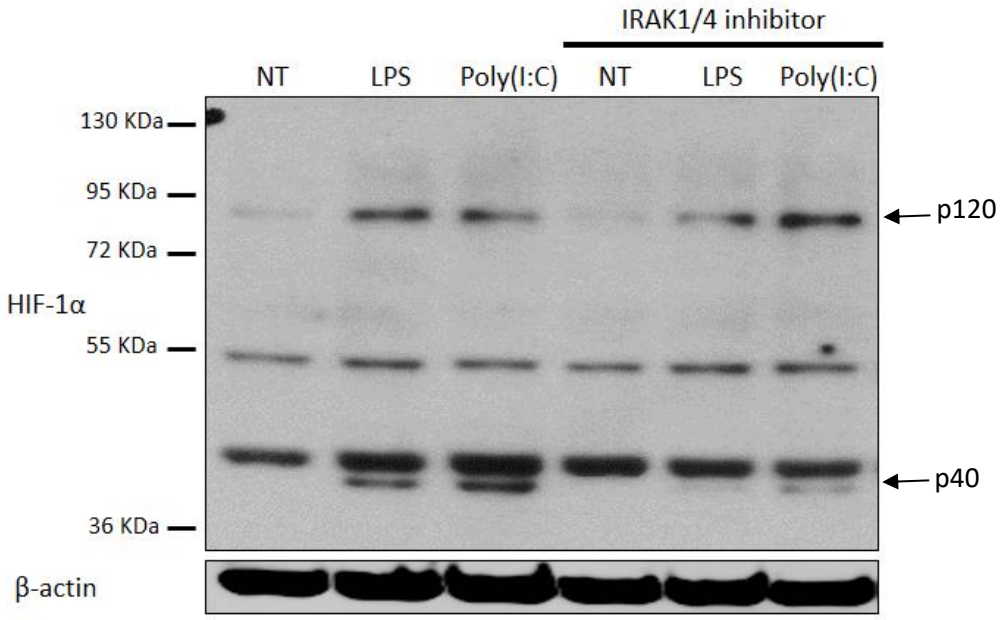

Figure 4.16 Inhibition of IRAK1/4 reduces TLR4/3-induced lower migrating form of HIF-1α in BMDMs.

Immunoblot analysis of HIF-1α (120 kDa) in cell lysates from WT BMDMs pre-treated for 1 hour with 20 μM IRAK1/4 inhibitor, followed by stimulation with 40 ng/mL LPS or 25 μg/mL Poly(I:C) for 6 hours. β-actin (42 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

results suggest TLR3/4-induced lower form of HIF-1α depends on downstream activation of their signalling pathways.

Having shown that the IRAK inhibitor affects the levels of the 40kDa migrating form of HIF-1α, studies next explored the role of the Pellino E3 ubiquitin ligases in regulation of HIF since Pellino proteins are substrates for IRAK kinase activity. Furthermore, our group has previously suggested that TNF receptor associated 6 (TRAF6) can mediate stabilisation of HIF-1α under conditions that simulate obesity and that the ubiquitin ligase Pellino 3 negatively regulates such TRAF6-induced HIF-1α stabilization in obesity-induced inflammation (Yang *et al.*, 2014). We were thus interested in exploring the wider role of Pellino 2 and Pellino 3 in TLR-induced regulation of HIF-1α. Since TRAF6 is employed by TLR4 but not TLR3 we were especially interested to evaluate the effects of Pellino deficiency on HIF-1α stabilization in response to LPS and Poly(I:C). WT, *Peli2*$^{-/-}$ and *Peli3*$^{-/-}$ BMDMs were treated with Poly(I:C), LPS or both for 8 hours (**Figure 4.17**). Phosphorylated TBK1 was used as an indicator of TRIF-dependent signalling (typical of TLR3 and TLR4 signalling). Again, in WT BMDMs both LPS and Poly(I:C) led to stabilisation of both the higher migrating form (110kDa) and the lower migrating form (40kDa) of HIF-1α. However, the expression levels of the 40 kDa form of HIF-1α in cells co-treated with both LPS and Poly(I:C) was slightly reduced compared to the levels of the same band in cells treated with either ligand alone. As previously described in our lab, *Peli3*$^{-/-}$ BMDMs treated with LPS showed increased expression of HIF-1α compared with WT BMDMs as evidenced by increased levels of the 110kDa band (Yang *et al.*, 2014). Interestingly, Pellino 3 deficiency did not affect the expression levels of the lower 40kDa band that was manifested by LPS. In addition, the levels of the 110kDa band were comparable in samples from WT and *Peli3*$^{-/-}$ BMDMs treated with Poly(I:C) suggesting that the negative effects of Pellino 3 on HIF-1α is restricted to the TLR4 pathway. This is hardly surprising since TLR3 does not employ TRAF6. The patterns of HIF-1α stabilised by LPS and Poly(I:C) in *Peli2*$^{-/-}$ BMDMs were largely the same as in WT cells. These data indicate that Pellino 3 negatively regulates TLR4- but not TLR3-induced stabilisation of HIF-1α whereas Pellino 2 appears to lack a role in regulating HIF-1α in these pathways.

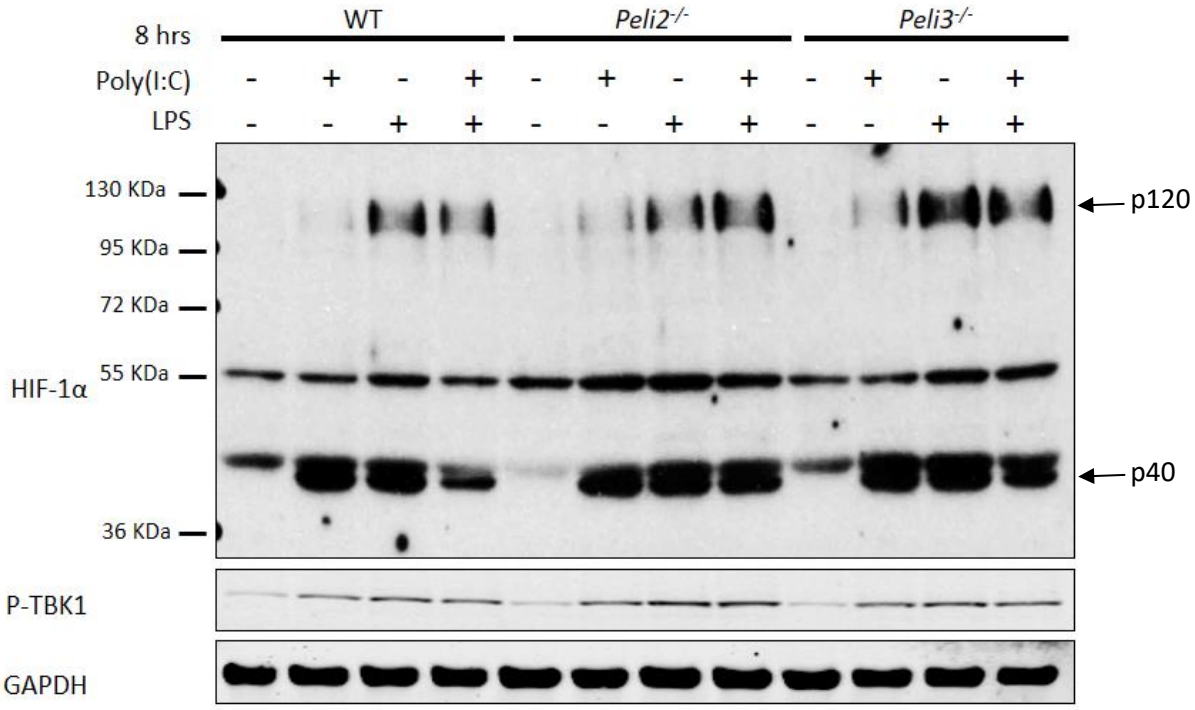

Figure 4.17 Differential roles for Pellino 2 and 3 in regulating TLR4/3-induced stabilisation of HIF-1α.

Immunoblot analysis of HIF-1α (120 kDa) and phosphorylated (p-) TBK1 (84 kDa) in cell lysates from WT, *Peli2*$^{-/-}$ and *Peli3*$^{-/-}$ BMDMs stimulated with 25 μg/mL Poly(I:C) and/or 40 ng/mL LPS for 8 hours. GAPDH (37 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

4.3 Discussion

HIF signalling has been shown to interact with inflammatory pathways at many levels. Several transcription factors, including NF-κB, STAT3 and AP-1 , essential in inflammation and innate immune response have been closely linked with HIF activation, (Alfranca *et al.*, 2002; Salnikow *et al.*, 2002; Jung *et al.*, 2005; Maxwell *et al.*, 2007; Bruning *et al.*, 2012; Pawlus, Wang and Hu, 2014). The present study evaluated the changes in HIF-1α expression induced by stimulation of different TLR signalling pathways. When a panel of six ligands for TLRs was evaluated, increased levels of HIF-1α was seen at the typical molecular size of 110kDa. This data is to be expected since TLR-induced NF-κB signalling has been shown to upregulate expression of HIF-1α (Rius *et al.*, 2008; van Uden, Kenneth and Rocha, 2008). However, in addition to the upper band, a lower form of HIF-1α of approximately 40kDa was observed when murine BMDMs were treated with Poly(I:C) or LPS (**Figure 4.3A**). This lower form of HIF-1α has not been previously described. Indeed, publications in the HIF field tend to show regions of immunoblots corresponding to mobilities of proteins of 75kDa and above. As TLR3 has been previously described to modulate a number of cellular processes through HIF signalling in inflammation and cancer, a potential isoform induced by this specific viral associated pathway was considered worthy of further characterisation. Interestingly, this lower form of HIF-1α was evident in response to treatment of cells with Poly(I:C) but not in response to hypoxic conditions (**Figures 4.3B and 4.4**). The nature of this lower form of HIF-1α is not yet understood. Two mRNA isoforms of HIF-1α have been described in mice, encoded from different promotors and exons: I.1 and I.2 (Wenger *et al.*, 1998; Lukashev *et al.*, 2001). The expression of I.1 has been described to be tissue specific being expressed highest in kidney, tongue, stomach, and testis in mice, while the I.2 isoform is ubiquitously expressed in all tissues (Wenger *et al.*, 1998; Paone *et al.*, 2010). It could be considered that one of these isoforms corresponds to the lower band observed in the macrophages treated with LPS or Poly(I:C). However, Lukashev and co-workers reported in 2001 that the main difference between the proteins encoded by the isoforms I.1 and I.2 is a loss of a 12 N-terminal amino acid residues in the protein encoded by I.1 (Lukashev *et al.*, 2001). Such a modest truncation would not explain the appearance of a protein of ~40kDa in size. Based on the results of this thesis, it cannot be ruled out that the 40kDa form of HIF-1α corresponds to a potential spliced form of one of these isoforms. However, further studies are required to address this possibility. It is to note that the lower band was only seen with the antibody from the company Novus, a polyclonal antibody that recognizes the region between residues 775 and the

C-terminus (residue 826) of HIF-1α (Novus NB 100-449; Species cross-reactivity: human, mouse, rat, chicken, monkey and non-human primate). The other three well-known and widely used antibodies from BD (CAT# 610958; Species cross-reactivity: human), Cell signalling (CAT# 14179; Species cross-reactivity: human, mouse, rat and monkey) and Abcam (CAT# ab16066; Species cross-reactivity: human, mouse, cow, pig, non-human primate) are monoclonal and correspond to residues 610-727 and residues surrounding Lys460 and 530-826 (C-terminal) respectively. The immunoreactvity of the faster migrating HIF form with an antibody raised against region 775-826 suggest the 40kDa protein is likely a C-terminal fragment of HIF-1α (**Figure 4.18**).

We explored the hypothesis that TLR signalling may promote the processing of full length HIF-1α into a smaller 40kDa form by assessing if TLR3 or TLR4 ligands could generate this smaller form from full length HIF-1α previously stabilised by the PHD inhibitor DMOG. However, both TLR agonists were unable to trigger such processing. We also examined if the putative processing of HIF-1α could be mediated by the proteasome or lysosomal-mediated degradation. However, the interpretation of these studies was complicated by ancillary effects of the inhibitors of these pathways. The use of the proteasome inhibitor MG132 in conjunction with the TLR ligands resulted in cell apoptosis and this is likely due to MG132 precluding IκB degradation and NF-κB activation thus preventing the induction of pro-survival proteins such as c-FLIP, IAP proteins and bcl proteins (Brasier, 2006; Gilmore, 2006). The lysosome inhibitor chloroquine inhibited the stabilisation of HIF-1α in response to Poly(I:C) but this does not necessarily relate to effects on the lysosome since all downstream TLR3 signalling was blocked by chloroquine. Instead the effects of chloroquine are likely due to inhibition of initial activation of TLR3 in the endosome.

Interestingly and unlike BMDMs, MEFS did not display the lower form of HIF-1α and did not undergo apoptosis in response to co-stimulation with LPS and MG132. Such findings may relate to different isoforms of HIF-1α being upregulated in a tissue-specific manner and a novel TLR-mediated mechanism of regulating macrophages' retraining or pattern of tolerance (Wenger *et al.*, 1998; Paone *et al.*, 2010; Butcher *et al.*, 2018). Indeed, HIF-1α may have specific roles in macrophages and this may underlie the close relationship of hypoxic and inflammatory signalling in the myeloid lineage (Cramer *et al.*, 2003; Palazon *et al.*, 2014). Upregulation of proinflammatory cytokines and chemokines or their corresponding receptors has been described to be a HIF/NF-κB-mediated mechanism activated by TLR signalling in different types of immune cells and diseases such as rheumatoid arthritis, sepsis and cancer (Maxwell *et al.*, 2007; Peyssonnaux *et al.*, 2007; Hu *et al.*, 2014; Matijevic Glavan *et al.*, 2017). There is also much

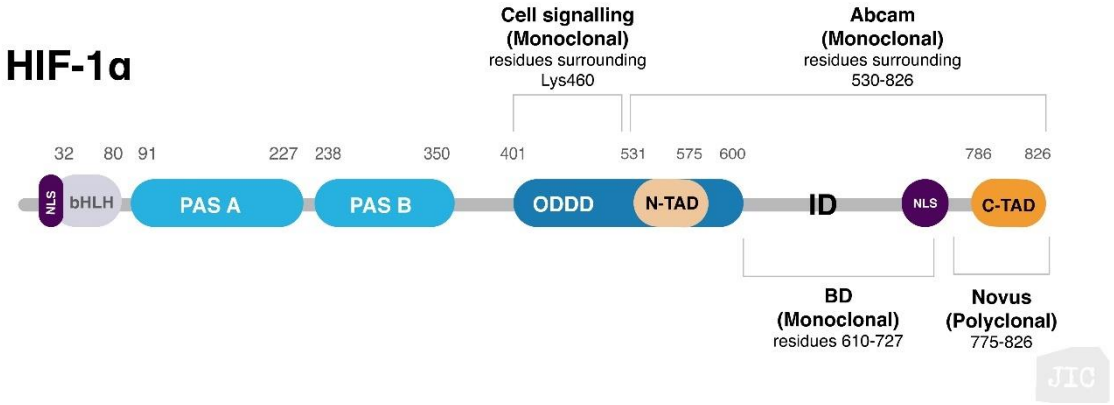

Figure 4.18 Schematic representation of the binding of different antibodies to HIF-1α.

The antibody used for this research work recognizes the region between residues 775 and 826 in the C-terminus of HIF-1α (Polyclonal, Novus NB 100-449). The other three well-known antibodies are monoclonal and correspond to residues 610-727 (BD, CAT# 610958) and residues surrounding Lysine 460 (Cell signalling, CAT# 14179) and 530-826 (C-terminal) (Abcam, CAT# ab16066).

interest in the importance of metabolic re-programming to glycolysis for inflammatory function in macrophages. HIF-1α plays a key role in this process by upregulation of glycolytic enzymes (Dengler, Galbraith and Espinosa, 2014). However, we now show that a glycolysis inhibitor can reduce levels of HIF-1α suggesting a regulatory loop between the HIF-1α pathway and glycolysis.

Efforts were also made to identify the receptor proximal signalling molecules that are used by TLRs to stabilise HIF-1α including its smaller 40kDa form. Immortalized BMDMs deficient for TRIF and IRF3 provided evidence of a TRIF/IRF3-dependant signalling axis upstream of HIF-1α stabilisation. This was especially true for the TLR3 pathway. We also further probed the role of TLR4 proximal molecules and demonstrated important roles for IRAK kinases but not for their E3 ubiquitin ligases Pellino 2 and Pellino 3. Such findings suggest that whilst both TLR3 and TLR4 can similarly regulate HIF-1α, the upstream signalling components that effect this common outcome differ between the 2 TLR pathways.

The functional relevance of HIF-1α to TLR signalling is likely to extend beyond effects on inflammation. Indeed, this relationship may have functional relevance to the metabolic and immunological reprogramming that favours the chemoresistance and tumour progression induced by TLR3 and TLR4 in different types of cancer (Salaun *et al.*, 2006; Paone *et al.*, 2010; Zhang *et al.*, 2010; Oblak and Jerala, 2011; Tewari *et al.*, 2012; Veyrat *et al.*, 2016; Matijevic Glavan *et al.*, 2017). In addition, HIF-2α has been shown to be overexpressed in tumour-associated macrophages (TAMs) and to upregulate proinflammatory cytokine/chemokines in LPS-activated macrophages *in vitro* (Imtiyaz *et al.*, 2010). This research also described that mice lacking HIF-2α in myeloid cells were resistant to LPS-induced endotoxemia and present less TAM infiltration in murine models of hepatocellular and colitis-associated colon carcinoma (Imtiyaz *et al.*, 2010). Evidently, understanding the innate immune signalling-mediated regulation of HIF-1α isoforms in macrophages could facilitate the targeting of these cells in pathological scenarios. Whilst the present studies add some insight into the mechanisms by which TLRs can trigger likely stabilisation of HIF-1α, they also highlight novel and additional complexity in that TLRs promotes the formation of smaller forms of HIF-1α than the classical 110kDa form. The dominant smaller form with mass of ~40kDa has not been previously described. It is likely to represent some C-terminal processed form or fragment of HIF-1α since it is recognised by a highly specific anti-HIF-1α antibody and is only evident when full length HIF-1α is also visible. **Table 4.2** summarises the findings of this research regarding the expression of HIF-1α lower form in the different types of cells evaluated. Further studies are required to identify this smaller form and its functional relevance.

Type of cells	Treatment	HIF-1α lower band (40kDa)
Primary BMDMs		
WT	LPS	++
	Poly(I:C)	+++
With IRAK1/4 inhibitor	LPS	NB
	Poly(I:C)	+
Immortalized BMDMs		
WT	LPS	+++
	Poly(I:C)	+
TRIF-deficient	LPS	NB
	Poly(I:C)	NB
IRF3-deficient	LPS	+
	Poly(I:C)	NB

Table 4.2 Summary of the observed expression of HIF-1α lower band (~40kDa) in primary BMDMs and immortalized BMDMs (WT, $TRIF^{-/-}$ and $IRF3^{-/-}$) treated with LPS or Poly(I:C). Conventions: +++ strong intensity of HIF-1α lower band; + lower intensity of the band; NB: No band.

Interestingly as part of these studies a very novel and unanticipated finding was observed when macrophages were treated with MG132 to explore the potential role of the proteasome in any putative processing of HIF-1α. Efforts were also made to correlate any effects on HIF-1α processing with expression of TLR-responsive cytokines. Intriguingly, as part of these approaches, it was noted that treatment of macrophages with MG132 alone induced high levels of IL-10. This highlighted a potential new relationship between the proteasome and anti-inflammatory IL-10 and warranted more detailed characterisation as described in the next chapter.

CHAPTER FIVE
IL10 EXPRESSION INDUCED BY PROTEASOME INHIBITION

5.1 Introduction

As previously described, the immune system has multiple ways of self-regulating its inflammatory responses to protect tissues and biological systems from excessive and prolonged activation of soluble or cellular pro-inflammatory signals. The cytokine IL10 is an important immunotolerant signal. This cytokine has an essential role in regulating the response of the immune system to pathogens and it is commonly induced by cells in the innate and adaptive immune system (Saraiva & O'Garra, 2010). However, increased levels of the IL10 in patients with cancer has also been described as an indicator of poor prognosis and disease progression, especially in the case of multiple myeloma (MM) (Mannino *et al.*, 2015). The proteasome inhibitor Bortezomib is one of the first line treatments for MM, but a number of patients have been shown to become refractory to the treatment. The reason for loss of responsiveness is not well understood. In the previous chapter, murine primary macrophages treated with the proteasome inhibitor MG132, produced high levels of IL-10. This raised the intriguing possibility that Bortezomib could also induce IL-10 in macrophages that would be counter-productive in its treatment of MM. This may have serious consequences in terms of its therapeutic efficacy and so the regulation of IL-10 by proteasome inhibitors was considered worth of further investigation in this study.

5.1.1 IL10 biology, cellular expression and signalling pathways

Interleukin (IL)-10 is one of the most studied anti-inflammatory soluble proteins. Its main function is to regulate excessive immune response to infectious pathologies and to restrict autoimmune responses. Structurally, human IL10 is a homodimer of subunits of approximately 35kDa (Zdanov *et al.*, 1995). Multiple single nucleotide polymorphisms (SNPs) have been described for the IL10 promoter and its receptor, some of them with strong correlations to cancer or autoimmune diseases (Jung *et al.*, 2016; Kasamatsu *et al.*, 2017). Most immune cells types have the capacity to produce IL10. Its upregulation by a particular kind of cell is specific to the nature of tissue affected, the stimulus being activated and the time of the immune response (Sabat *et al.*, 2010). As reviewed by Saraiva and O'Garra in 2010, IL-10 is largely derived from monocytes-macrophages, dendritic cells (DCs), mast cells, natural killer (NK) cells, neutrophils and eosinophils in the innate immune system. Additionally, many T lymphocytes subpopulations have been identified as producers of IL10, among them T helper 1 (T_H1), T_H2 and T_H17, as well as T_{Reg}, CD8+ lymphocytes and B cells in the adaptive immune system (**Figure 5.1**). In cells from the innate system, production of IL10 seems to regulate excessive activation to exogenous

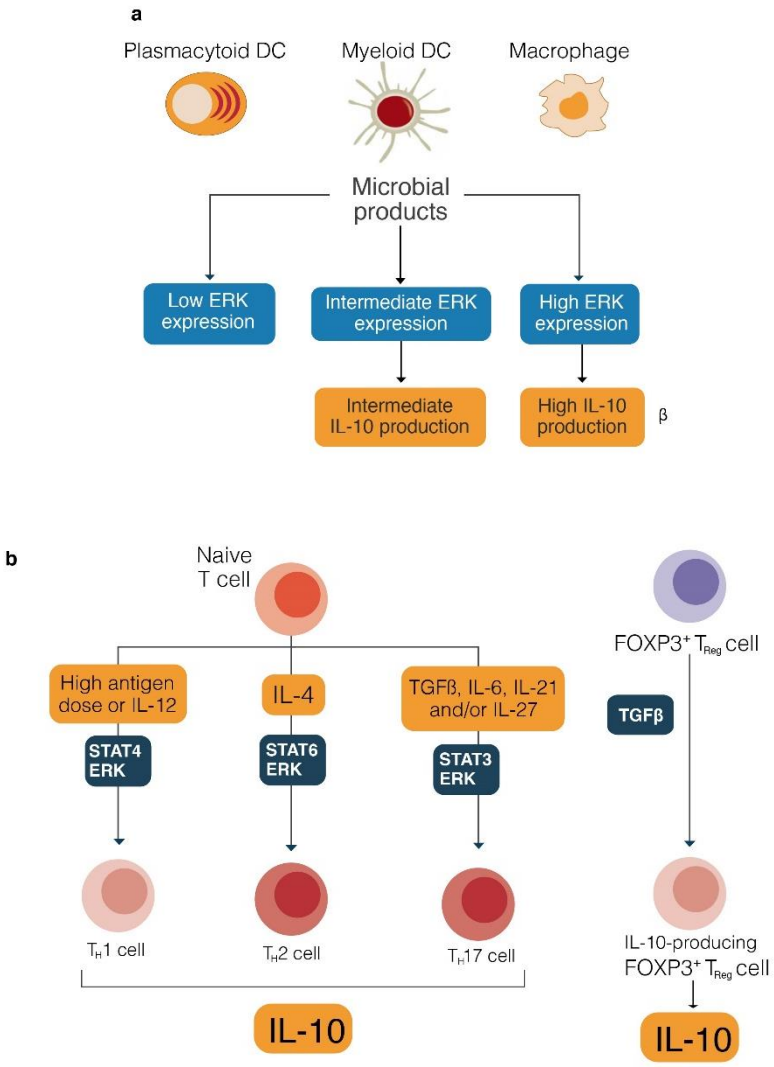

Figure 5.1 IL10 expression by immune cells.

(A) Macrophages and myeloid dendritic cells (DCs), produce IL10 in response to microbial products mostly through ERK-dependent signalling. Meanwhile, production of IL10 by **(B)** T helper (Th) cells requires of a specific profile of cytokines expression depending on the cell subtype. Differentiation and downstream signalling activation of STAT and ERK pathways is necessary for production of IL10 in naive CD4+ T cells. The Th1 subtype depends on high doses of antigen presented by DCs and/or IL-12 to differentiate from naïve cells and produce interferon-γ (IFNγ) or IL10. IL-4 and STAT6 signalling pathways are necessary for Th2 cells to produce IL10. In the case of Th17 the signalling mechanism is not clear but is believed to depend on TGFβ, IL6, IL-21 and/or IL-27 and STAT3 signalling. Other cells like T_{Reg}, CD8+ T cells, B cells, mast cells and eosinophils, also produce IL10 (Adapted from Saraiva & O'Garra, 2010).

pathogens or endogenous signals from apoptotic cells by limiting T_H1 and T_H2 responses and upregulating differentiation of T_{Reg} cells. In addition, IL10 produced by T helper 1 (T_H1), T_H2 and T_H17 cells negatively regulates the activation of DCs and macrophages (Saraiva and O'Garra, 2010).

Macrophages are a rich source of IL10 especially in response to ligands which are recognized by TLR2, TLR3, TLR4 and TLR9 (Boonstra *et al.*, 2006; Saraiva and O'Garra, 2010; Teixeira-Coelho *et al.*, 2014). IL10 can also regulate different functions of macrophages. IL10 can enhance macrophage-induced immunotolerance by inhibiting production of pro-inflammatory soluble mediators like TNF-α, IL-1β, IL6, IL-8, G-CSF, and GM-CSF, and by limiting antigen presentation (Malefyt *et al.*, 1991; Sabat *et al.*, 2010). At the same time, IL10 boosts the release of anti-inflammatory molecules such as IL-1 receptor antagonist and soluble TNFα receptors (Sabat *et al.*, 2010).

TLR-dependant IL10 production in macrophages depends on downstream activation of both MyD88 and TRIF signalling pathways (Boonstra *et al.*, 2006). Activation of P38 and N-terminal kinase (JNK) MAPK signalling cascades mediated by MyD88 and its associated mediators IL-1R-associated kinase and TNF receptor- associated factor 6 (TRAF6) has been broadly described (O'Neill, Golenbock and Bowie, 2013). In addition, extracellular signal-regulated kinases (ERK), another essential MAPK protein, has also been shown to be strongly activated in response to TLRs stimulation through TPL-2 signalling (Kaiser *et al.*, 2009). Continuous phosphorylation of P38 and ERK, or their chemical inhibition or deficiency in cells has been proven to regulate the respective upregulation or downregulation of IL10 production in activated T helper cells and macrophages (Chi *et al.*, 2006; Saraiva *et al.*, 2009). P38 and ERK act by stimulating the downstream kinases MSK1 and MSK2 which will induce phosphorylation of transcription factors CREB and AP1, and their consequent binding to IL10 and DUSP1 gene promoters (Ananieva *et al.*, 2008; Wen, Sakamoto and Miller, 2010). Once produced and released, IL10 binds to its receptor IL10R and exerts its function through Janus kinase (JAK)1 and STAT3 by triggering the anti-inflammatory cascade (Lang *et al.*, 2002; Murray, 2006) (**Figure 5.2**).

IL10 can also engage in self-regulatory pathways. On one side, CREB or STAT3-dependant production of DUSP1 can negatively regulate P38 activation and consequently IL10 (Hammer *et al.*, 2005; Ananieva *et al.*, 2008). Furthermore, IL10R dependant activation of STAT3 induces TPL-2 expression and a positive feedback loop of activation by means of ERK (Lang *et al.*, 2002). IFNγ also plays an important role in the regulation of IL10 expression. It can directly

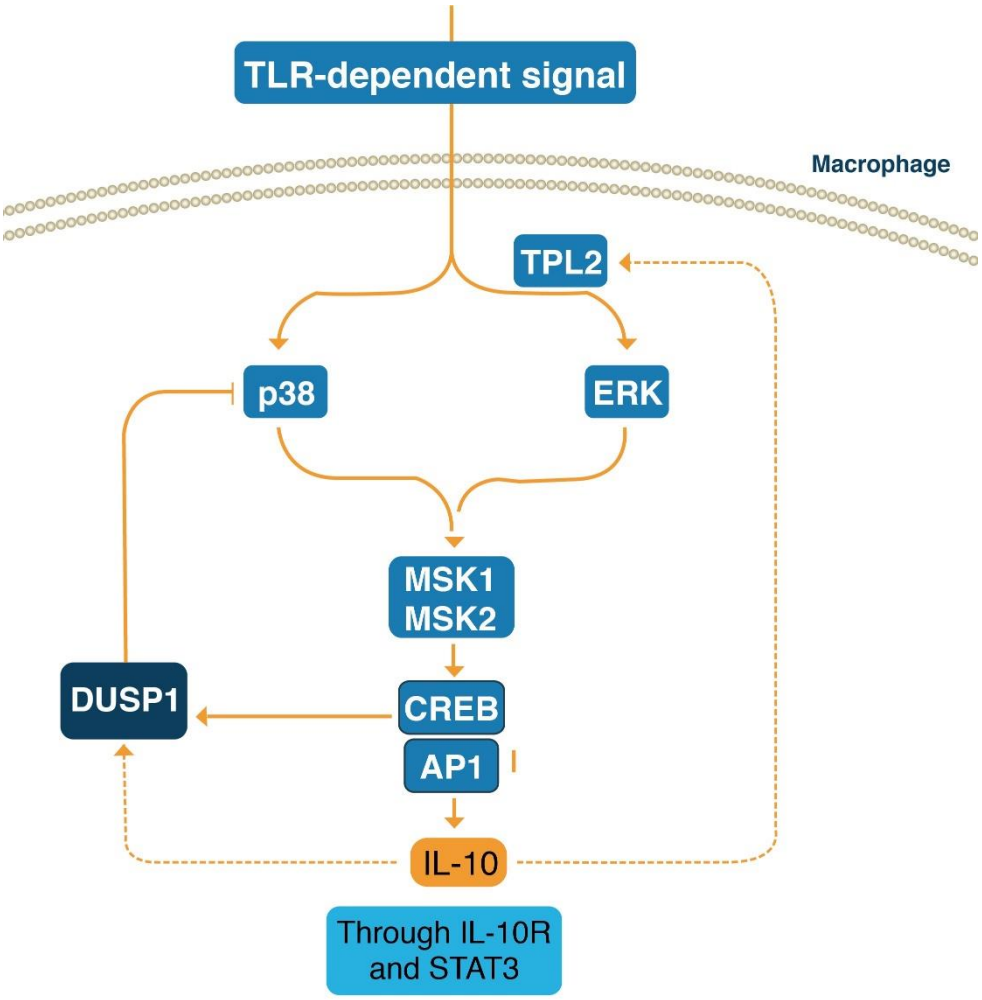

Figure 5.2 TLRs signalling and IL10 production in macrophages.

Activation of MAPKs, p38 and ERK, downstream TLRs signalling cascades has been shown to induce IL10 expression in a signalling dependent on MSK1/MSK2, and the transcription factors CREB and AP1 in macrophages. The production of IL10 in this context is regulated by interferon-γ (IFNγ) and IL10 itself. Expression of dual-specificity protein phosphatase 1 (DUSP1) induced by IL10 negatively regulates p38 phosphorylation and consequently IL10 production mediated by this MAPK. IL10 can also upregulate its own expression by inducing tumour progression locus 2 (TPL2) and ERK phosphorylation (Adapted from Saraiva & O'Garra, 2010).

inhibit activation of ERK and P38 MAPKs decreasing CREB activity or induce GSK3 which blocks the transcription factors CREB and AP-1 (Hu *et al.*, 2006; Wen, Sakamoto and Miller, 2010).

Considering its crucial role in immunotolerance, IL10 overexpression or downregulation has been associated with the pathogenesis of multiple diseases. An increase of the cytokine can promote the progression of some stablished infections or some types of cancer like B/T/NK-cell lymphomas, melanoma and lung cancer (Mannino *et al.*, 2015). Meanwhile, IL10 deficiency is generally associated with pathologies where the immune system is over activated like Crohn's disease or rejection of organ transplantation. It has also been associated with multiple autoimmune disease like psoriasis and rheumatoid arthritis (Sabat *et al.*, 2010; Mannino *et al.*, 2015). In the interest of this thesis, we will focus on the role of IL10 in a type of B cell lymphoma named multiple myeloma.

5.1.2 Multiple myeloma (MM)

5.1.2.1 Multiple myeloma

Multiple myeloma is a type of mature B cell neoplasm lymphoma derived from plasma cells (NCCN Foundation, 2018). It represents approximately 10% of all lymphomas and 1% of all neoplasm diseases. MM has been described to be more common at aged 65 and over, and two times more frequent in African-Americans than Caucasians (Rajkumar, 2016). One of the main characteristics of the disease is bone damage, but fatigue, weight loss, anemia, renal failure, and infections have also been described as clinical symptoms (Rajkumar, 2016; NCCN Foundation, 2018). MM can be subdivided into five subtypes depending on cytogenetic characteristics of the disease, with trisomic multiple myeloma and IgH translocated multiple myeloma being the most frequent (Rajkumar, 2016). Additionally, deletions in chromosomes 1p, 17p and 13, gains in 1q, mutations in MYC, RAS and proteins in the NF-κB pathways, as well as Ig translocations, have been associated with poor prognosis of MM (Chesi *et al.*, 2014). These cytogenetic abnormalities are essential in driving disease progression, but also influence the response to treatment and prognosis.

A combination of drugs is used as standard treatment for MM, with thalidomide, Bortezomib and lenalidomide being the most commonly used (Palumbo and Rajkumar, 2009). Many others have been recently approved for treatment of relapsed patients, including carfilzomib, pomalidomide, panobinostat, ixazomib, elotuzumab, and daratumumab (Rajkumar, 2016). Overall, thalidomide, lenalidomide, and pomalidomide act mainly by inducing cytotoxicity (Wells, Parman and Wiley, 1999), while Bortezomib, carfilzomib, and ixazomib are proteasome inhibitors which initiate cell arrest and apoptosis in cells with high rates of defective proteins, as is the case with malignant plasma cells in MM (Kubiczkova *et al.*, 2014; Rajkumar, 2016). Other drugs like Elotuzumab, daratumumab and panobinostat act by targeting specific characteristic signals or mechanisms particular to malignant proliferation in MM and are mainly used in patients who have relapsed (Anderson, 2016; Rajkumar, 2016). Regardless the combination of drugs used, many patients become resistant or have relapses after the treatment. Therefore, studies have focussed in understanding the immunomodulatory mechanism that can be affecting the efficacy of treatment to improve patient's survival rate.

5.1.2.2 MM and IL10

Among the potential immune molecules and signalling mechanisms studied in correlation to MM progression, IL10 production and its polymorphisms have been extensively investigated. Pappa and co-workers described in 2007 a significant positive correlation between cell proliferation and high levels of IL10 in serum of non-treated MM patients when compared with healthy controls (Pappa *et al.*, 2007). A positive correlation between IL10 and different markers of angiogenesis like vascular endothelial growth factor (VEGF) and angiopoietin-2 (ANG-2), as well as proliferation markers have also been reported in serum from MM patients in a more recent publication (Alexandrakis *et al.*, 2015). In the same sense, increased serum levels of IL10 were also correlated to the stage of disease, with MM stage III patients expressing more IL10 compared with stage I patients (Shekarriz, Janbabaei and Kenari, 2018). Similar results were showed in a human cell line of MM named RPMI 8226, where IL-4 and IL10 were increased together with the chemokines CXCL12, CCL5, MIP-1β, and CXCL10 (Freire-de-Lima *et al.*, 2017). Moreover, single-nucleotide polymorphism (SNP) of both IL10 and its receptor have been shown to be associated with drug resistance, specifically to treatment with thalidomide and/or Bortezomib, and in general to poor prognosis in MM (Kasamatsu *et al.*, 2017; Nielsen *et al.*, 2017). All this evidence suggests IL10 to play a role in the pathogenesis and progression of MM, and it may also be relevant for diagnosis and defining clinical treatment. However, further studies are necessary to fully understand how IL10 is induced and how to restrict its effects in treatment.

5.1.3 Proteasome inhibition in cancer treatment

As previously mentioned, Bortezomib is one of the classic treatments for MM and two more proteasome inhibitors have been recently approved as new generation drugs, carfilzomib, and ixazomib. Degradation of proteins mediated by the proteasome is an essential mechanism of regulation at cellular level. Therefore, its inhibition can significantly impact cell viability and potentially trigger immunoregulatory mechanisms in biological systems. Hereby, the role of proteasome inhibitors in the context of immunity and cancer will be explored.

5.1.3.1 Proteasome biology

Protein production is a central process in all types of cells and due to its essential role, it's a highly regulated one. The ubiquitin proteasome system is one of the main mechanisms used by eukaryotic cells to control quality and concentration of proteins in cell. It also mediates the inactivation of biological signalling pathways by degrading active proteins once they are no longer required. The proteasome is the core effector of this system. It is a proteolytic complex of high molecular weight (2.5MDa) which recognizes mainly ubiquitin targeted proteins and cuts them into small peptide chains of 8-12 amino acids (by Inobe & Matouschek, 2014).

Structurally, the proteasome has been described as a cylindrical complex made primarily of two subunits known as 20S core particle (CP) and 19S regulatory particle (RP) which together form what is known as the 26S proteasome (**Figure 5.3**) (Murata, Yashiroda and Tanaka, 2009; Kish-Trier and Hill, 2013). The core particle includes 28 subunits arranged in four rings of 7 units each, two external rings made of α-units and two internal rings made of β-units. The proteolytic active sites of the proteasome are in the inner space of both β-rings, and α-rings act as gateways to the proteolytic chamber. Subunits β1, β2 and β5 of 20S in eukaryotes have been described to have hydrolytic caspase, trypsin, and chymotrypsin-like activity, respectively (Murata, Yashiroda and Tanaka, 2009). Inhibition of the proteasome proteolytic rings is a key target for therapeutic studies in cancer treatment, as it will be described later in this section. In addition to the well-known proteasome, a specialised immunoproteasome that is induced by IFN-γ or type I interferons has been identified in hematopoietic cells. The immunoproteasome is made of specific catalytical subunits similar to the constitutive β1, β2 and β5 known as low molecular mass polypeptide 2 (LMP2 or β1i), multicatalytic endopeptidase complex-like 1 (MECL-1, or β2i), and LMP7 (β5i), which are integrated in de novo proteasomes (Griffin *et al.*, 1998; Khan *et al.*, 2001; Shin *et al.*, 2006). It is generally accepted immunoproteasomes produce hydrophobic C-terminus peptides that are to be presented by MHC class I molecules (Gaczynska *et al.*, 1994; Toes *et al.*, 2001).

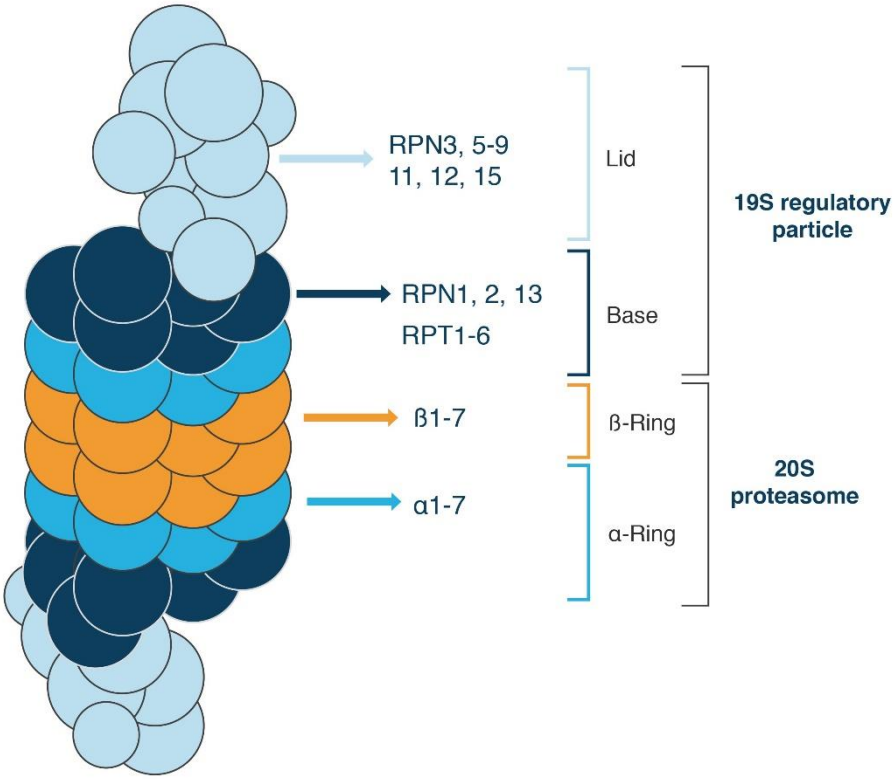

Figure 5.3 Schematic representation of 26S proteasome structure.

The 26S proteasome consists of a 20S core particle and 19S regulatory particles. The different colors indicate the subunit composition for the 26S proteasome: 20S is made of four rings: two outer α-rings and two inner β-rings (catalytic subunits), and 19S which is subdivided in base (triple-A or RPT subunits) and lid (non-ATPase or RPN subunits) subcomplexes (Adapted from Murata, Yashiroda and Tanaka, 2009).

The second major structural component of proteasomes is the regulatory particle 19S. It is known to be the portion responsible for recognizing, binding and delivering the proteins labelled to be degraded. 19S is ATP-dependent and it contains 19 subunits arranged in two subcomplexes usually described as the base and lid that can be attached to one or both ends of the core particle. The base contains six ATPases (Rpt1–6), two large subunits (Rpn1 and Rpn2), and two ubiquitin receptors Rpn10 and Rpn13. Those subunits allow the identification and binding of proteins carrying polyubiquitylated chains, and their unfolding to enter through the α-rings. Nine non-ATPase subunits (Rpn3, 5–9, 11, 12, and 15) integrate the lid of 19S complexes, from which Rpn11 it is known to have deubiquitylase activity and mediates de-ubiquitylation of proteins before their degradation (Kish-Trier & Hill, 2013; Murata et al., 2009). Understanding the structure and function of the proteasome will allow a better understanding of its relevance in the context of cancer therapy and the mechanistic understanding of clinical treatments based on proteasome inhibition, which will be expanded next. As mentioned, for the proteins to be recognized and degraded by this system, a polyubiquitin chain most be attached to them. The mechanism by which the proteins are labelled was discussed in detail in Section 3.1.3.

5.1.3.2 Proteasome inhibitors in cancer therapy: Bortezomib

Cancer cells are often characterized as having multiple alterations from single nucleotide mutations to major changes at chromosomal level. It is to be expected that such changes would affect protein quality and concentrations in malignant cells, and furthermore transformed cells would be more dependent on proteasomal efficiency (**Figure 5.4**) (Deshaies, 2014). In this sense, Torres and co-workers showed in 2007 that proliferation of aneuploidy yeast was hampered using the proteasome inhibitor MG132 (Torres *et al.*, 2007). This and previous evidence on cancer research using proteasome inhibitors in cancer cell lines supported induction of the so called proteotoxic crisis as a clinical approach to cancer treatment (Deshaies, 2014; Teicher & Tomaszewski, 2015). This strategy suggests that, by using inhibitors of the protein quality control system within the cells, effector mechanism of homeostasis (as cell death) could be activated in response to accumulation of toxic signals (Manasanch *et al.*, 2014). The proteotoxic crisis approach seems to be more effective in cancers derived from mature B lymphocytes such as multiple myeloma and mantle cell lymphoma, perhaps due to their high rate of protein production in the form of immunoglobulins.

Given the essential role of the proteasome system in cellular biology, using molecules that could inhibit its activity as a therapeutic approach was not considered possible at first. However, research on the effect of proteasome inhibitors in cancer cell lines and mouse tumour

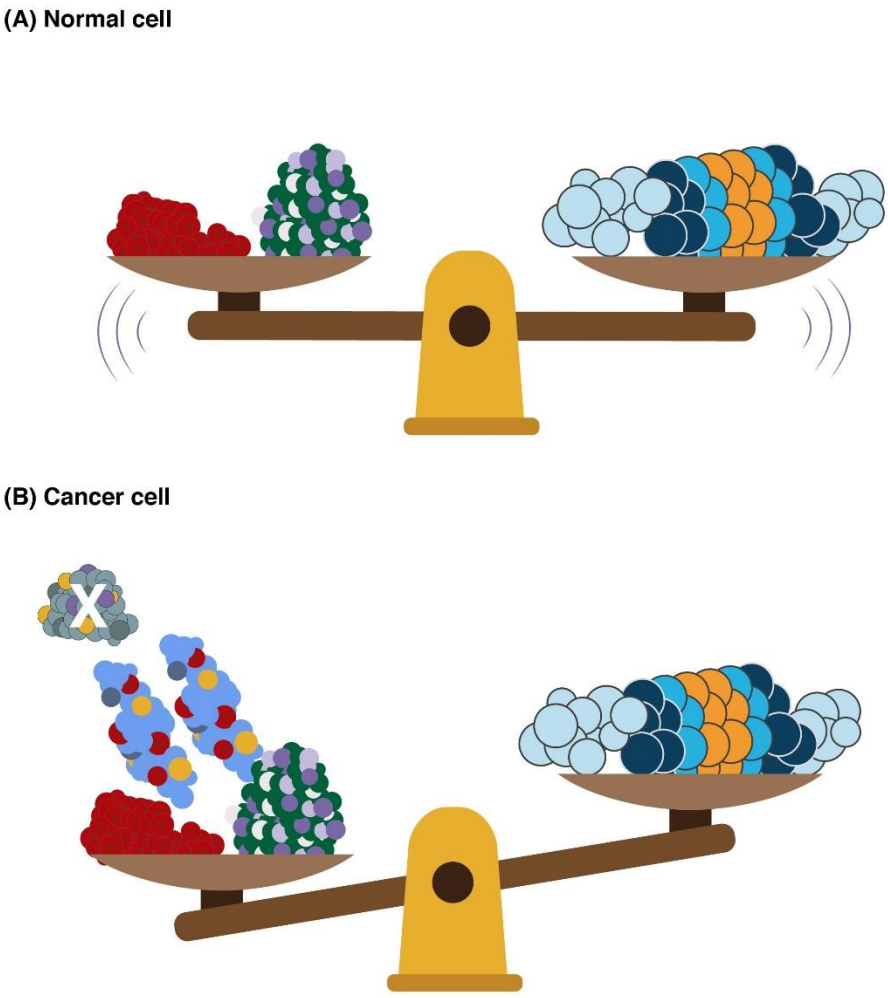

Figure 5.4 Proteotoxic crisis in cancer cells.

(A) It is expected the rate of protein production (to the left) and protein degradation by the proteasome (represented to the right) would be in balance in normal cells. However, in cancer cells **(B)**, it is believed this balance is altered due to overexpression of proteins or their mutant versions what would affect degradation efficiency by the ubiquitin proteasome system (UPS) inducing the so-called proteotoxic crisis (Adapted from Deshaies, R. 2014).

models began in the early 90s with a compound called PS-341, also known as Bortezomib (Teicher & Tomaszewski, 2015). This boronic acid inhibitor binds in a reversible way mainly to β5-subunits in the 20S portion of the proteasome blocking its chymotrypsin activity. Subsequently, multiple *in vitro* studies showed MM cell lines and patients samples were more sensitive to apoptosis, and their proliferation was inhibited in the presence of this proteasome inhibitor (Hideshima *et al.*, 2001; Ma *et al.*, 2003). Based on these and other findings, Bortezomib became the first inhibitor to be approved as a frontline treatment for MM, relapsed/refractory MM and mantle cell lymphoma in the past fifteen years (Buac et al., 2013; Teicher & Tomaszewski, 2015).

Treatment with Bortezomib alone or in combination with other immunomodulatory drugs (like thalidomide and lenalidomide) increased the overall survival rate for MM patients up to three times. Nevertheless, the treatment has not proved to be effective in solid tumours and at least one third of the patients have been reported not to respond or to develop resistance to treatment (Ping Dou and Zonder, 2014; Moreau *et al.*, 2015). In addition, it has been suggested that Bortezomib treatment induces permanent peripheral neuropathy (PN) in some patients (Argyriou *et al.*, 2008). In light of these evidence, a second generation of proteasome inhibitors (Carfizomib, Ixazomib, Delanzomib, Oprozomib and Marizomib) has been approved or are under study for clinical use (Ping Dou and Zonder, 2014). In addition, recent findings have shed light on why Bortezomib might not be effective in some cases. On one side, it has been shown in preclinical models for immunological disorders like lupus erythematosus (SLE), myasthenia gravis (MG) and autoimmune haemolytic anaemia that treatment with proteasome inhibitors downregulates short- and long-lived plasma cells (Škrott and Cvek, 2014). It is suggested the efficiency of treatment with proteasome inhibitors is enhanced in types of cancer where the protein production rate is also high, as it would be the case of MM, a plasma cell-derived malignancy. Furthermore, Pitcher and co-workers have proved that nano molar concentrations of Bortezomib induced up to 95% inhibition in chymotrypsin-like active sites and structural changes of the proteasome specifically in MM cells compared to other cancer cells, which would trigger a proteotoxic crisis (Pitcher *et al.*, 2015).

Multiple approaches have been explored to tackle Bortezomib-resistance and increase its efficiency in treatment. Identification of cytogenetic abnormalities like 1q21 gain as a biomarker correlated to Bortezomib resistance and poor survival were described in high-risk MM patients (Wu *et al.*, 2018). These findings show the importance of personalized treatment based on specific risk factors for each MM patient. Multiple studies have focused their efforts in testing combined treatment of Bortezomib with other cancer drugs like lenalidomide,

dexamethasone and Ibrutinib, or with small molecules to overcome patients relapsed or refractory response to treatment with the proteasome inhibitor (Richardson *et al.*, 2014; Murray *et al.*, 2015; Siegel *et al.*, 2015). However, Bortezomib treatment and direct induction of IL10 production in MM has not been associated. As part of a screening of multiple inhibitors in Chapter Four of this thesis, a proteasome inhibitor MG132 was shown to induce IL10 in murine macrophages. This would appear to be highly counterproductive for its treatment of MM which is driven by IL10 so further studies were performed to better understand the regulation of IL10 by proteasome inhibitors.

5.2 Results

5.2.1 Proteasome inhibition by MG132 induces IL10 expression in BMDMs

Proteasome inhibition by Bortezomib is a widely used clinical approach for the treatment of Multiple Myeloma (MM), mantle cell lymphoma, and some child autoimmune diseases (Buac *et al.*, 2013; Khandelwal *et al.*, 2014; Kouroukis *et al.*, 2014; Škrott and Cvek, 2014). As described in the previous chapter, anti-inflammatory cytokine IL10 expression was increased in macrophages treated with MG132, another known proteasome inhibitor more commonly used as a research tool to study proteasome biology. Considering the correlation of IL10 with progression and poor prognosis of MM, and that proteasome inhibitors are important in the treatment of this disease, the potential role of the inhibitors in inducing the anti-inflammatory cytokine was contemplated. To address this question, expression of cytokines IL10 and IL6 was assessed in supernatants of BMDMs treated with MG132 for 1h and then stimulated for 24hrs in the absence/presence of the TLR4-ligand LPS (**Figure 5.5**) or TLR3-ligand Poly(I:C) (**Figure 5.6**). When cells were treated with LPS, both cytokines, IL6 and IL10, were significantly induced and their expression was reduced when cells were pre-treated with MG132 before stimulation with LPS. However, IL10 was induced by MG132 treatment alone when compared with non-treated cells (**Figures 5.5A and 5.6A**). MG132 alone failed to induce significant levels of IL6 suggesting some specificity for induction of IL10. To further explore the degree of specificity of this response, the effects of MG132 on the expression of pro-inflammatory chemokines CXCL1 and CXCL2 were also measured (**Figures 5.5B and 5.6B**). LPS induced high levels of both CXCL1 and CXCL2 which were reduced in the presence of MG132. Interestingly MG132 alone induced some modest basal expression of CXCL2 whereas CXCL1 expression remained undetectable. These results suggest that the proteasome inhibitor MG132 strongly induces the anti-inflammatory cytokine IL10 with more modest effects on CXCL2 expression without affecting the expression of other pro-inflammatory cytokines like IL6 or CXCL1.

The kinetic profile of MG132-induced expression of IL10 was next characterised in more detail. IL10 was first detected at 8h post MG132 challenge and increased strongly up to 24hrs (**Figure 5.7A**). The mRNA levels of *Il10* at the same time points were next measured to assess if MG132 could promote the transcriptional up-regulation of *Il10*. When assessed for IL10 mRNA levels at the same time points, a modest 7-fold rise was observed at 4 hours with further 60-fold and 100-fold increments at 8h and 24h (**Figure 5.7B**). These data indicate a direct effect of MG132 on the transcriptional upregulation of IL10.

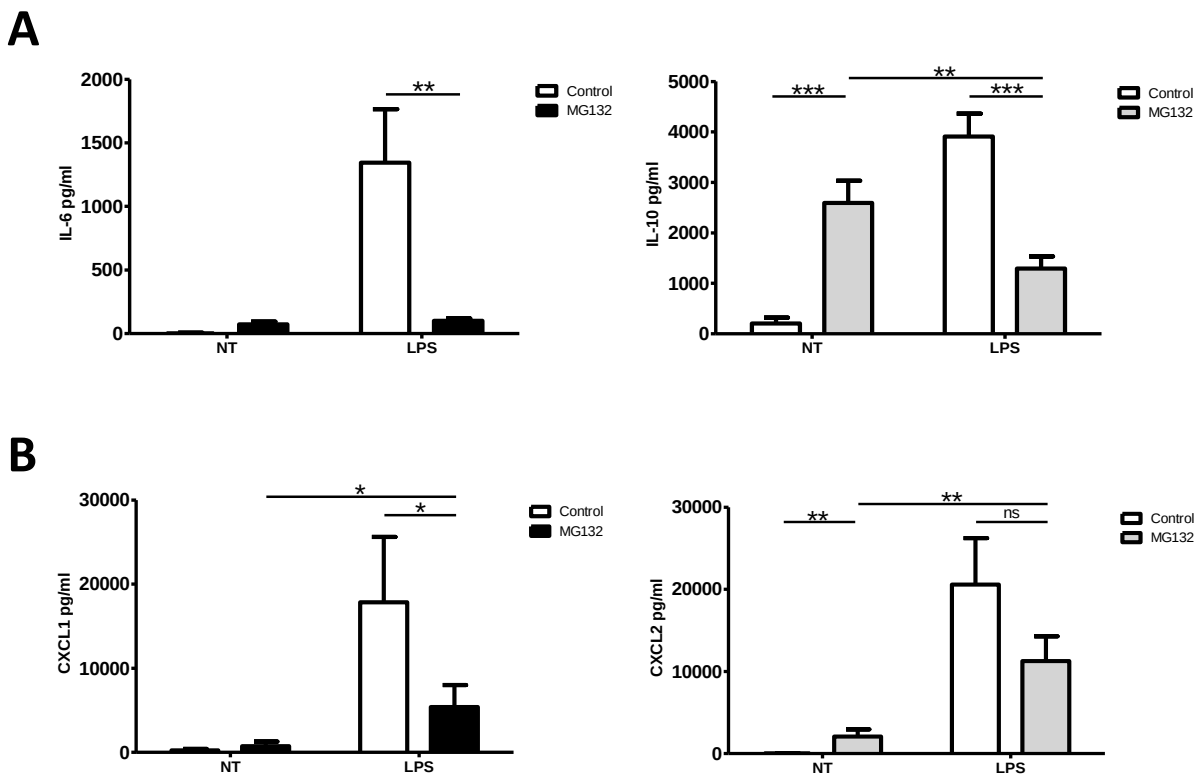

Figure 5.5 Effect of MG132 and LPS treatment on cytokine and chemokines expression in BMDMs.

ELISA analysis of **(A)** cytokines IL6 and IL10, and **(B)** chemokines CXCL1 and CXCL2 in supernatant of BMDMs pre-treated for 1h with 20 µM MG132, and subsequently stimulated for 24 hrs with 40 ng/mL LPS. Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. ns: not significant, *p < 0.05, **p < 0.001, ***p < 0.0001.

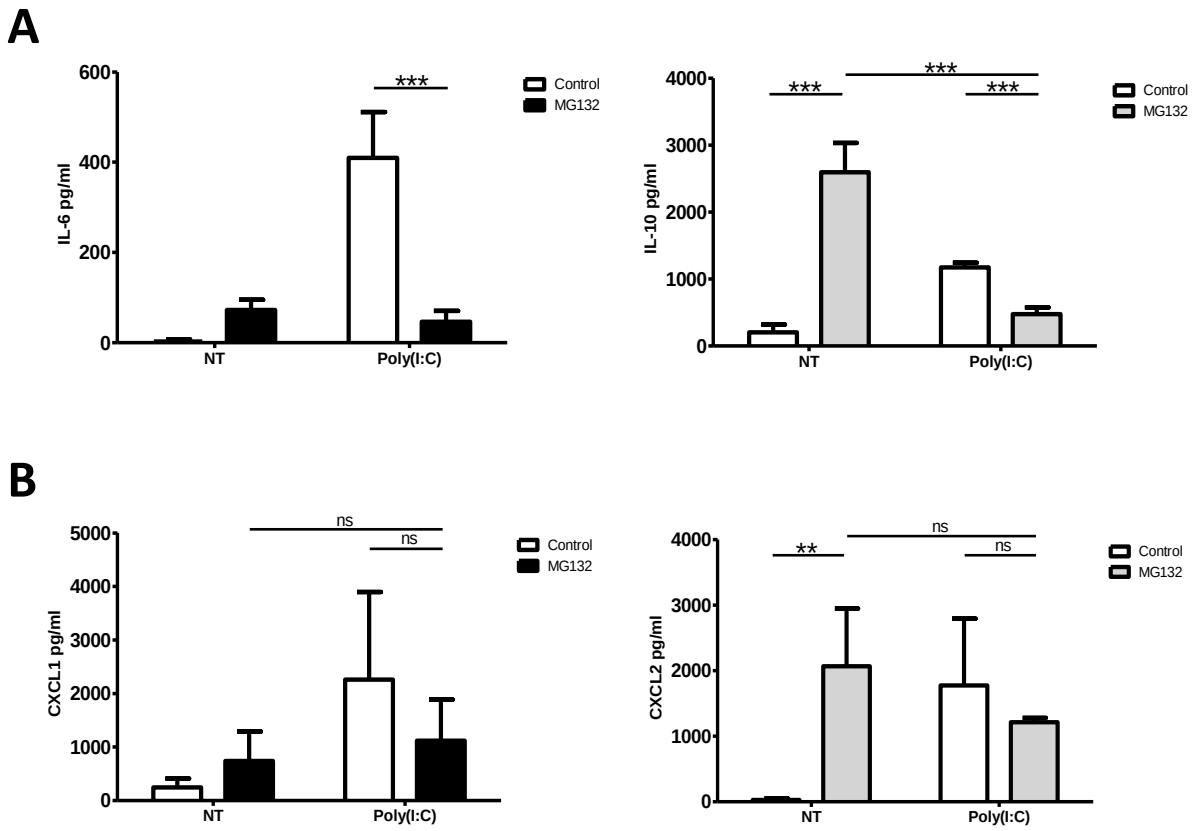

Figure 5.6 Effect of MG132 and Poly(I:C) treatment on cytokine and chemokines expression in BMDMs.

ELISA analysis of **(A)** cytokines IL6 and IL10, and **(B)** chemokines CXCL1 and CXCL2 in supernatant of WT BMDMs pre-treated for an hour with 20 µM MG132, and subsequently stimulated for 24 hrs with 25 µg/mL Poly(I:C). Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. ns: not significant, *p < 0.05, **p < 0.001, ***p < 0.0001.

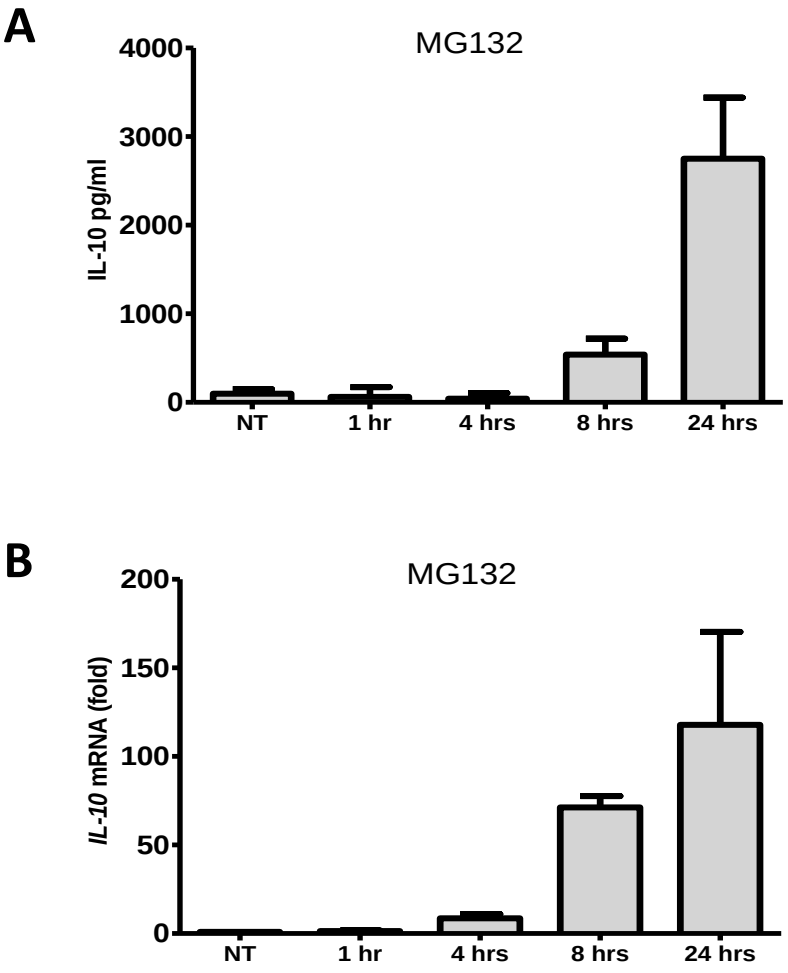

Figure 5.7 Inhibition of the proteasome with MG132 induces IL10 expression at protein and mRNA levels after 4 hours treatment.

(A) ELISA analysis of IL10 and **(B)** quantitative PCR of mRNA expression for IL10 in supernatants and cell lysates of WT BMDMs treated at different times with 20 µM MG132. Data are presented as the mean ± SEM of four (A) and two (B) independent experiments.

The next study aimed to address if the stimulatory effects of MG132 on IL10 expression is restricted to macrophages a cell type well known to be abundant in tumour microenvironment, and usually described as a facilitators of malignant cell migration and metastasis (Balkwill, Capasso and Hagemann, 2012). To determine the cell specificity of IL10 induction by MG132, murine derived dendritic cells (BMDCs) and a human monocytic cell line THP-1 were evaluated following the conditions previously employed with BMDMs (**Figure 5.8**). Protein levels of IL10 and CXCL2 were measured in both cell types after 1-24hrs of MG132 treatment. Neither BMDCs or THP-1 cells showed a significant increase in the expression of IL10 in the presence of the proteasome inhibitor MG132 at any of these time points. These data suggest that the stimulatory effects of MG132 on IL10 expression may be restricted to macrophages.

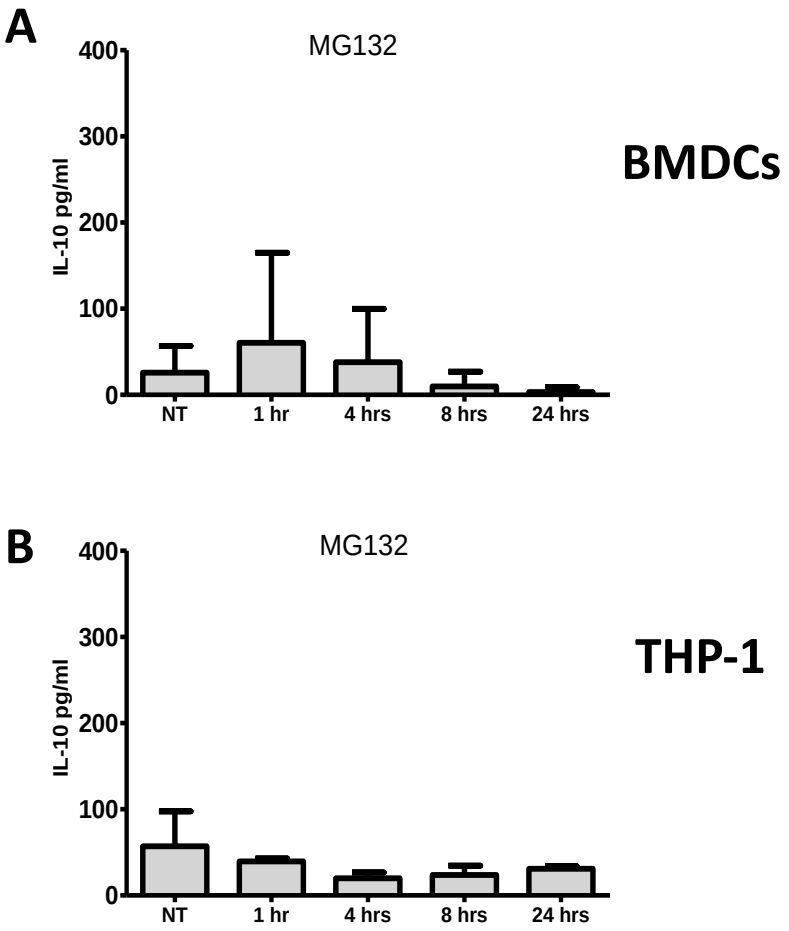

Figure 5.8 MG132 does not induces IL10 expression in dendritic cell or monocytes.

ELISA analysis of IL10 in supernatants of **(A)** WT BMDCs and **(B)** THP-1 cells treated at different times with 20 µM MG132. Data are presented as the mean ± SEM of three independent experiments.

5.2.2 p38 and JNK MAPKs mediate MG132 induced expression of IL10

Given IL10 expression was induced specifically in BMDMs and the response seems to be initiated within the first 4 hours of proteasome inhibition with MG132, studies next explored the mechanism underlying this induction. Macrophages are part of the first line of response in innate immunity, and one of the most important signalling pathways activated through TLRs is the activation of mitogen activated protein kinases (MAPKs) (Saraiva & O'Garra, 2010). Research from our group has previously showed that P38 signalling pathway is essential for the induction of IL10 (Mellett *et al.*, 2011).Therefore, activation of the three main kinases of this signalling pathway, P38, JNK and ERK, were measured in response to MG132 (Chi *et al.*, 2006; Ananieva *et al.*, 2008; Saraiva *et al.*, 2009; Wen, Sakamoto and Miller, 2010). In addition, the transcription factor CREB which also has an important role in activation of IL10 was examined (Wen, Sakamoto, & Miller, 2010). Activation of these proteins was assessed by evaluating their phosphorylation status, using phospho-specific antibodies, in cell lysates from BMDMs treated for different times with MG132. Phosphorylated forms of P38 and JNK were not detected in untreated cells but were strongly induced in response to MG132 at 30 min and remained sustained for up to 6 hours. (**Figure 5.9**). On the contrary, ERK (also known as P44/42) was present in its phosphorylated forms in untreated cells but diminished with increasing time exposure to MG132. These data clearly indicate that MG132 induces activation of P38 and JNK MAPKs but suppresses the ERK pathway in the BMDMs. Since MG132 promotes the transcriptional upregulation of IL10, we next measured its ability to promote activation of CREB, a key transcription factor that induces transcription for the *Il10* gene. Using phosphorylation of CREB as an index of its activation, MG132 was shown to promote time-dependent phosphorylation of CREB with a kinetic profile that closely mirrored the activation of P38 and JNK.

Studies next addressed if these MAPKs are part of the underlying mechanism mediating the expression of IL10 in response to MG132. To this end, selective inhibitors of the P38 and JNK pathways were used to block their activation. BMDMs were pre-treated with AMG548 and AEG3482 (inhibitors for P38 and JNK respectively) for 1 h and later stimulated with MG132 for 24 hours. IL10 and CXCL2 expression were then measured in the supernatant of the cells. Both inhibitors, when used individually or combined, suppressed the MG132-induction of IL10 (**Figure 5.10A**). However, the P38 inhibitor AMG548 was more effective than the JNK inhibitor AEG3482 in inhibiting IL10 expression. Indeed AMG548, alone or in combination with AEG3482, abrogated the ability of MG132 to induce IL10. The MAPK inhibitors showed similar suppressive effects on MG132 induced expression of the chemokine CXCL2, with a robust reduction when

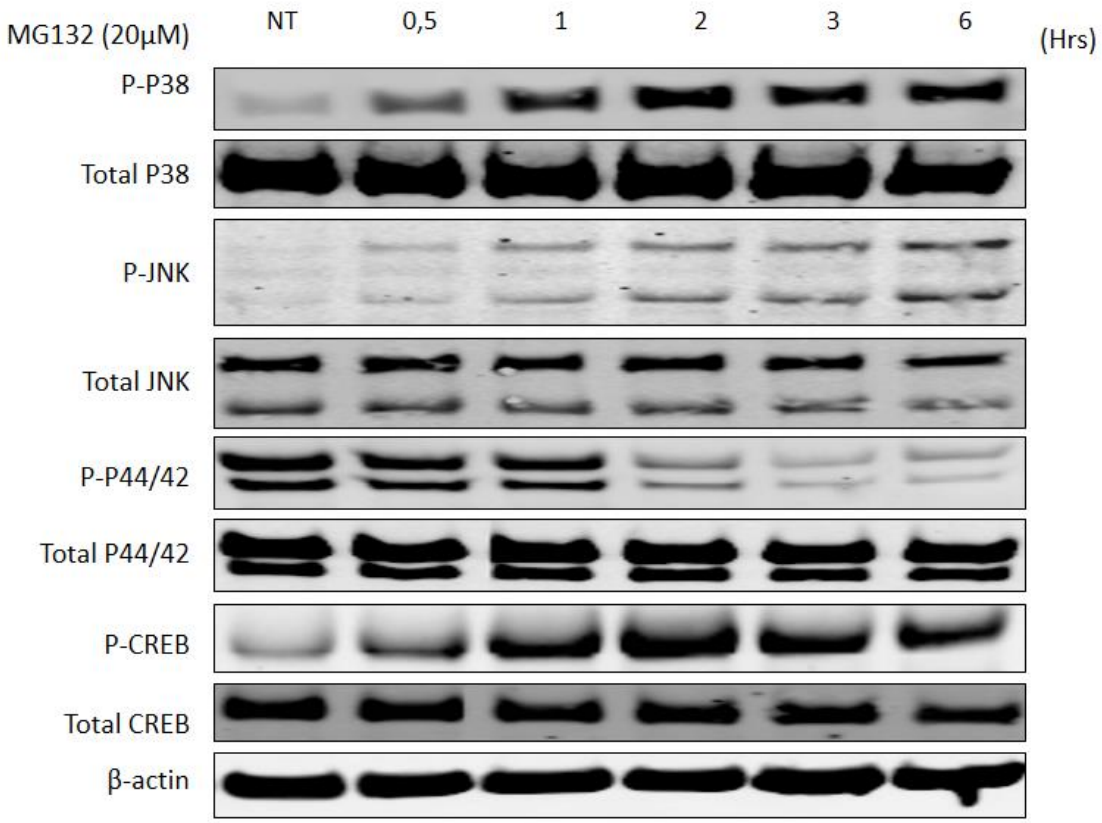

Figure 5.9 Proteasome inhibitor MG132 induces phosphorylation of P38 and JNK.

Immunoblot analysis of phosphorylated (p-) and total P38 (43 kDa), JNK (46-54 kDa), P44/42 (42-44 kDa) and CREB (43 kDa) in cell lysates from WT BMDMs treated at different times with 20 μM MG132. β-actin (42 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

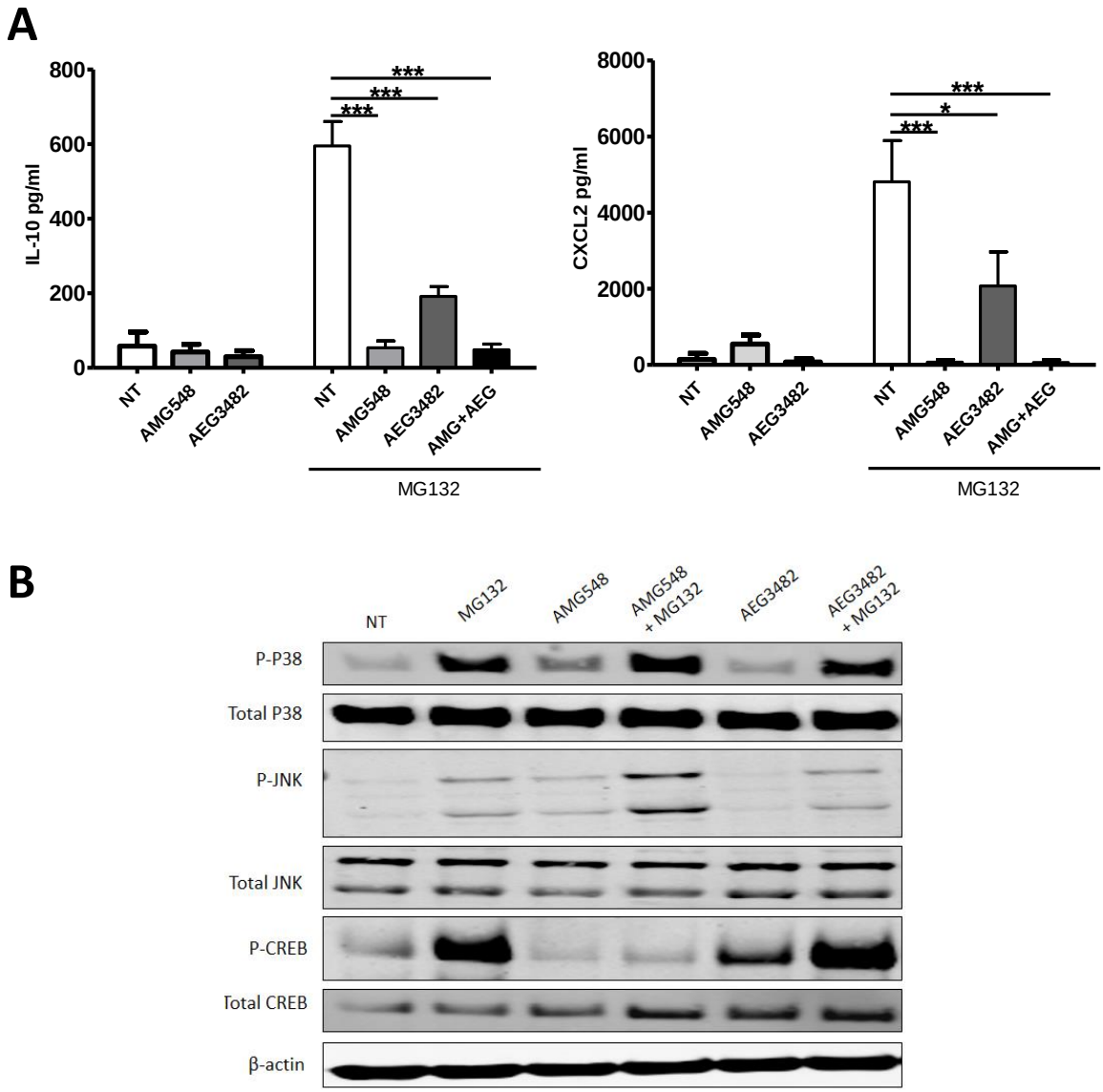

Figure 5.10 Inhibition of P38 and JNK reduces the expression of IL10 and CXCL2 induced by MG132.

(A) ELISA analysis of IL10 and CXCL2 in supernatants of WT BMDMs pre-treated with 10µM AMG548 (Inhibitor of p38, iP38) and/or 10µM AEG3482 (Inhibitor of JNK, iJNK) for one hour, and then treated with 20µM MG132 for 24 hours. **(B)** Immunoblot analysis of phosphorylated (p-) and total P38 (43 kDa), JNK (46-54 kDa) and CREB (43 kDa) in cell lysates from WT BMDMs pre-treated with 10µM AMG548 or 10µM AEG3482 for one hour, and then treated with 20µM MG132 for 2 hours. β-actin (42 kDa) was used as constitutively expressed housekeeping protein. Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

the inhibitor of P38 was used alone or in combination to JNK inhibitor, and a more modest decrease in the presence of the JNK inhibitor alone. In order to relate the suppressive effects of AM548 and AEG3482 on IL10 expression to regulation of p38 and JNK respectively, levels of total and phosphorylated P38, JNK and CREB were measured in cell lysates of BMDMs previously treated for 1 h with the corresponding inhibitor and for two hours with MG132 (**Figure 5.10B**). Proteasomal inhibition with MG132 induced the phosphorylation of P38, JNK and CREB. When the inhibitor AM548 was used combined with MG132, phosphorylation of P38 and JNK was again observed. It is to notice that phosphorylation of JNK was stronger in this case compared to phosphorylated JNK in cells treated with MG132 alone. However, phosphorylation of CREB was lost with AMG548 and MG132. These data are consistent with a role for p38 in mediating phosphorylation of CREB. AMG does not affect phosphorylation of p38 and this is an expected result considering it inhibits p38 kinase activity by competing with ATP (Zhang, Shen and Lin, 2007). The JNK inhibitor AEG3482 does not affect the ability of MG132 to promote phosphorylation of CREB. These results clearly prove that the proteasome inhibitor MG132 induces production of IL10 and CXCL2 in a P38 dependant manner that is likely mediated by the transcription factor CREB.

5.2.3 Bortezomib induces IL10 expression

Considering Bortezomib is the proteasome inhibitor used in clinical treatments for cancer and autoimmunity, its effect on the expression of IL10 and CXCL2 was also assessed to compare its efficacy with MG132. This may be of relevance to MM since IL10 is a growth factor for the myeloma and any macrophage expression of IL10 in response to Bortezomib may underlie the resistance of patients with MM to Bortezomib. To assess this, BMDMs were treated with different concentrations of Bortezomib for 24 hours and IL10 and CXCL2 expression at protein level were determined. IL10 expression was induced in the macrophages at concentrations as low as 0,5 µM and a clear dose-dependent increase was observed with concentrations up to 10 µM (**Figure 5.11A**). Since Bortezomib promotes cell death in myeloma cells, its cytotoxic effects on macrophages was also measured over the same concentration range as above. The percentage of cytotoxicity in BMDMs treated with Bortezomib was determined by means of analysis of LDH release (**Figure 5.11B**). Bortezomib caused 10% cytotoxicity at its lowest concentration and this increased to a maximum of 45% at highest concentrations. These results showed that Bortezomib can trigger the production of IL10 and this is also coincident with its induction of cell death.

Studies next addressed the signalling cascades being activated by Bortezomib. Phosphorylation status of P38, JNK and ERK (P44/42) MAPKs, and the transcription factor CREB were evaluated in response to Bortezomib (Chi *et al.*, 2006; Ananieva *et al.*, 2008; Saraiva *et al.*, 2009; Wen, Sakamoto and Miller, 2010). Activation was determined using phospho specific antibodies in cell lysates from BMDMs treated with 10µM Bortezomib treatment at different time points. Phosphorylated P38 and CREB were induced in response to Bortezomib at 30 minutes, reached their highest expression between 1 and 2 hours, and showed a moderately decrease after 3 hours of treatment. Whereas phosphorylation of ERK (also known as P44/42) was evident in untreated cells but progressively lost with time exposure to the same proteasome inhibitor (**Figure 5.12**). The reduction on phosphorylated P38 and CREB after 3 hours treatment with Bortezomib is most likely due to the reversible binding effect of this inhibitor to the proteasome (Teicher and Tomaszewski, 2015). Interestingly, JNK activation was not induced as strongly as with MG132, with Bortezomib only inducing a moderate phosphorylation of JNK after 6 hours.

Aiming to corroborate these observations, the selective inhibitors for P38 and JNK pathways were used to block their activation in response to Bortezomib. As previously described, BMDMs were pre-treated with either AMG548 (inhibitor for P38) or AEG3482

(inhibitors for JNK) for 1 h and later stimulated with Bortezomib for 24 hours. The expression of IL10 was then determined in the supernatant of the cells. Bortezomib-induction of IL10 was depleted completely by P38 inhibitor AMG548 when used individually or combined with AEG3482 (**Figure 5.13**). However, the JNK inhibitor AEG3482 failed to inhibit IL10 expression in response to Bortezomib. Evidently, proteasome inhibitor Bortezomib induces IL10 production in a P38 dependent signalling activation.

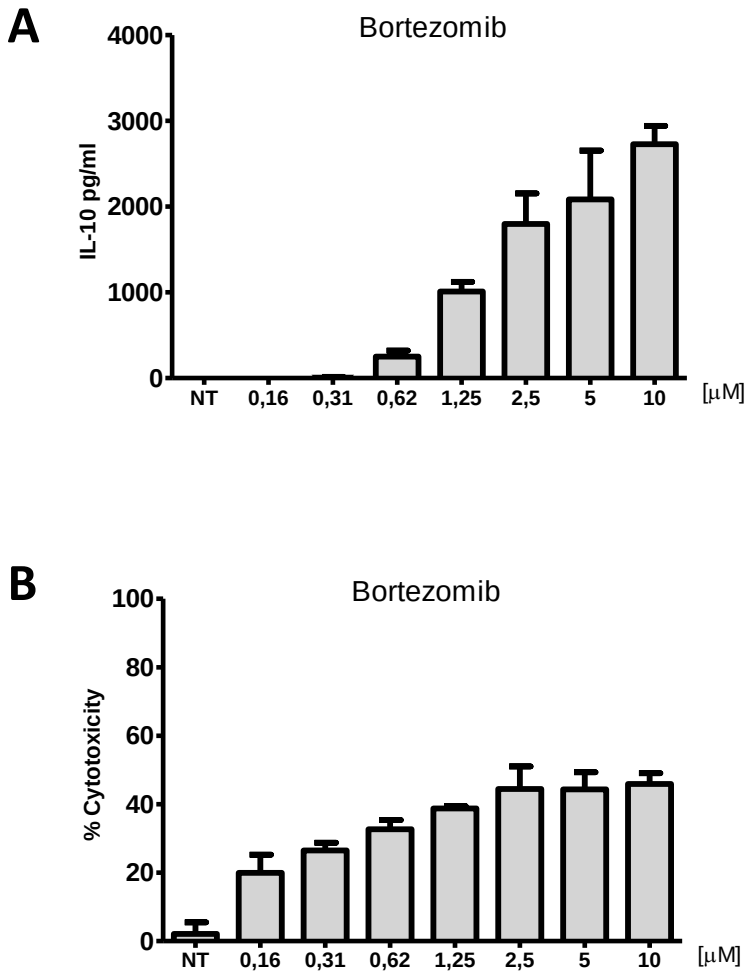

Figure 5.11 Bortezomib induces the expression of IL10 and increases cytotoxicity in BMDMs.

(A) ELISA analysis of IL10 and **(B)** cytotoxicity analysis by LDH release in supernatants of WT BMDMs treated with different concentrations of Bortezomib for 24 hours. Data are presented as the mean ± SEM of three independent experiments.

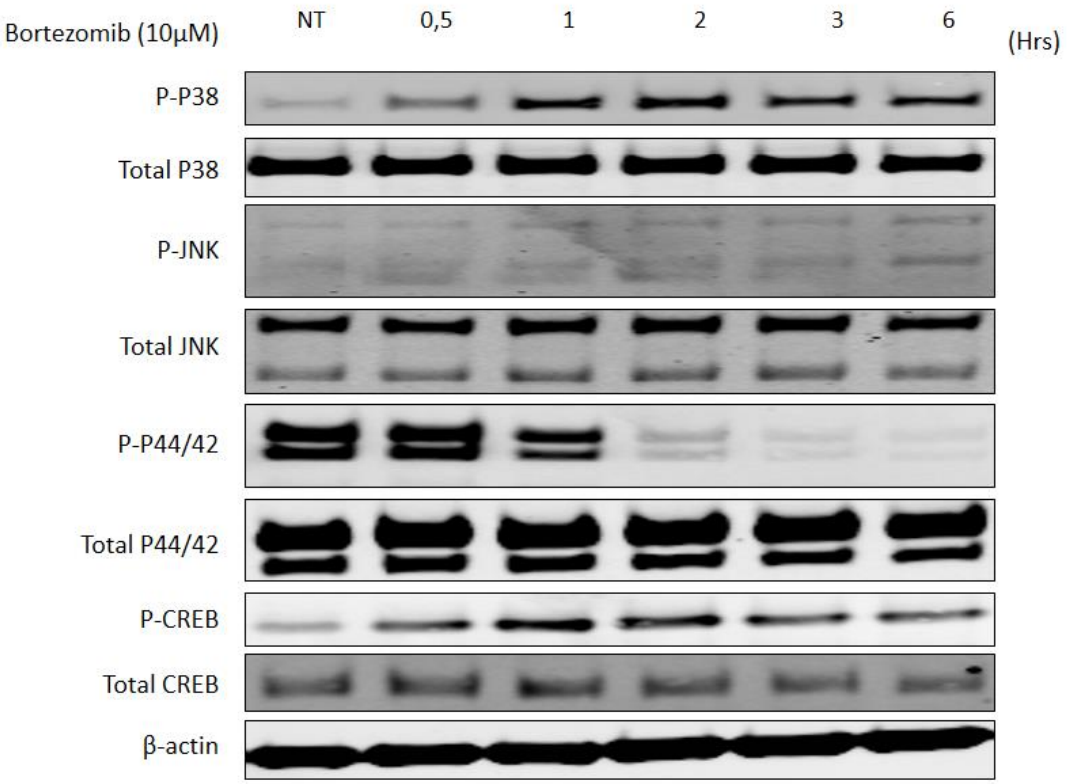

Figure 5.12 Proteasome inhibitor Bortezomib induces phosphorylation of P38 and JNK.

Immunoblot analysis of phosphorylated (p-) and total P38 (43 kDa), JNK (46-54 kDa), P44/42 (42-44 kDa) and CREB (43 kDa) in cell lysates from WT BMDMs treated at different times with 10 μM Bostezomib. β-actin (42 kDa) was used as constitutively expressed housekeeping protein. Data are representative of three experiments.

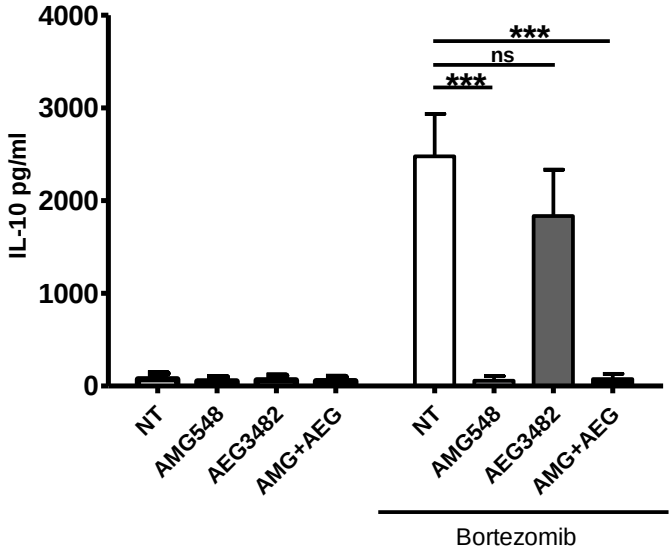

Figure 5.13 Inhibition of P38 reduces the expression of IL10 induced by Bortezomib.

(A) ELISA analysis of IL10 in supernatants of WT BMDMs pre-treated with 10µM AMG548 (Inhibitor of p38, iP38) and/or 10µM AEG3482 (Inhibitor of JNK, iJNK) for one hour, and then treated with 10µM Bortezomib for 24 hours. Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

5.2.4 Cell death and IL10 expression

Considering Bortezomib is inducing cell death in macrophages at concentrations that also induce IL10 the next studies investigated if the expression of IL10 was a key signature of macrophage cell death. Three forms of cell death were evaluated. The first one, pyroptosis is generally associated with cellular membrane rupture and release of Damage-associated molecular pattern (DAMPs) signals, such as IL-1β, that triggers a robust inflammatory response. Caspase-1 is the key mediator of this type of cell death and facilitates the activation of IL-1β and Gasdermin D, the main effector molecules (Broz & Dixit, 2016; Kolb, Iii, Oberst, & Martinez, 2017). The model of two signals for the activation of the inflammasome previously used in our lab was applied (Humphries *et al.*, 2018). IL10 and CXCL2 expression was assessed in BMDMs treated with LPS alone for 3 hours or LPS plus ATP for one additional hour. Both IL10 and CXCL2 were induced to the same levels by LPS alone or in combination with ATP (**Figure 5.14**). However, since pyroptosis requires both signals with LPS being insufficient, these results suggest that pyroptosis does not promote IL10 expression or secretion.

In addition, apoptosis and necroptosis were also studied in the context of IL10 expression following the conceptual and technical approaches described in chapter three of cell death from this thesis. Necroptosis, like pyroptosis, is usually described as a pro-inflammatory type of cell death while apoptosis is in general terms associated with immunotolerance. As described before, the pancaspase inhibitor Z-VAD and IAP inhibitor (LCL161) were used to drive the activation of these two types of cell death in the context of TNFα and TLR signalling. The combined treatment of LCL161 and TNFα or TLR ligands will promote apoptosis whereas the addition of Z-VAD to this dual treatment will favour necroptosis. The expression of IL10 was evaluated in supernatant of BMDMs in response to either TNFα, LPS or Poly(I:C) (**Figure 5.15A**). TNFα was unable to induce IL10 expression in the presence of Z-VAD, LCL161 or both, meaning this ligand does not promote expression of the anti-inflammatory cytokine in either apoptosis or necroptosis. Meanwhile, stimulation of the cells with LPS and either Z-VAD or LCL161 induces a moderate increase of the cytokine compare to LPS treatment alone, but it was significantly reduced with both inhibitors and the TLR4-ligand. These means IL10 can be induced in apoptosis but it is inhibited in necroptosis in response to bacterial infections. In the case of TLR3 activation, IL10 expression was induced by Poly(I:C) but this was lost when combined with inhibitors.

As for the expression of the chemokine CXCL2 in apoptosis and necroptosis, interestingly it was considerably induced by the combination of Z-VAD and LCL161 without ligands (**Figure 5.15B**). The expression of the chemokine in the context of TNFα signalling increased when TNFα

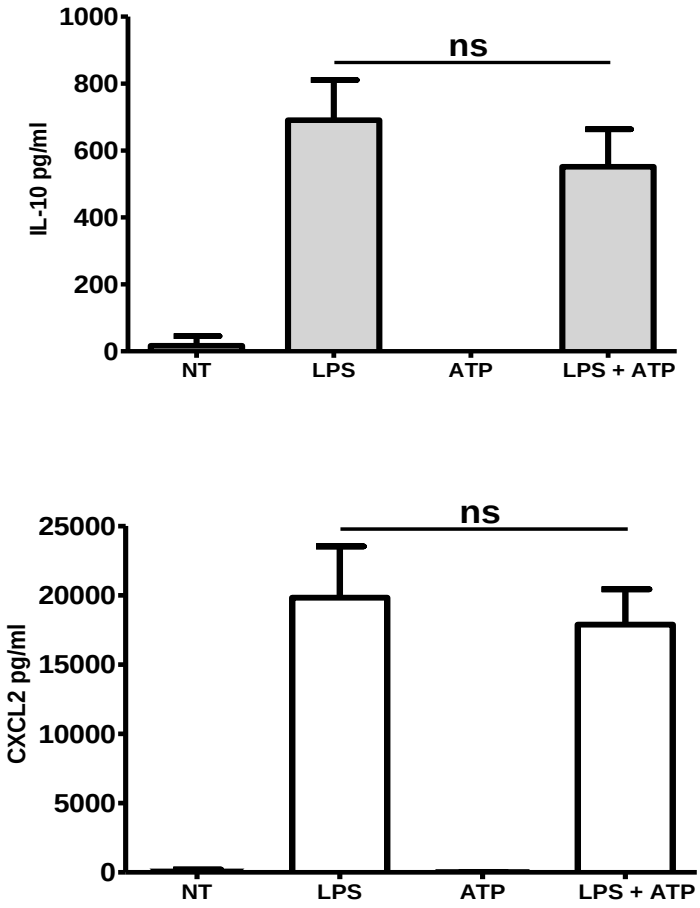

Figure 5.14 IL10 expression is not integral part of pyroptosis.

ELISA analysis of IL10 and CXCL2 in supernatants of WT BMDMs treated with 100 ng/mL LPS for 3 hours followed by 2,5mM ATP for 1 hour. Data are presented as the mean ± SEM of four independent experiments and were subjected to two-tailed Student's t-test. ns, not significant.

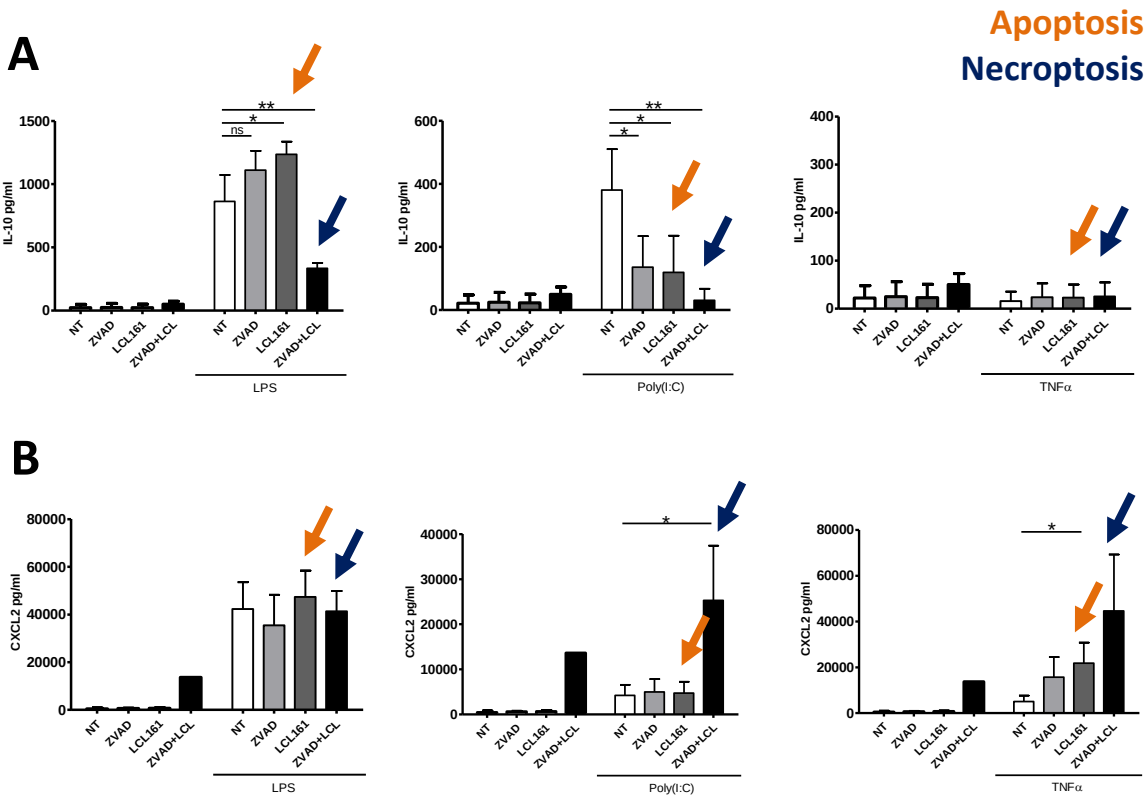

Figure 5.15 Expression of IL10 under conditions of apoptosis and necroptosis.

ELISA analysis of **(A)** IL10 and **(B)** CXCL2 in supernatants of WT BMDMs treated with 20 µM Z-VAD and 10 µM LCL161 for an hour and then stimulated with 100 ng/mL LPS, 25 µg/mL Poly(I:C) or 40 ng/mL TNFα for 24 hrs. Data are presented as the mean ± SEM of three independent experiments and were subjected to two-tailed Student's t-test. ns: not significant, *p < 0.05, **p < 0.001, ***p < 0.0001.

was used with either Z-VAD and LCL161 or their combination, indicating that pro-apoptotic or pro-necrotic signals activate CXCL2. This chemokine was greatly induced by LPS stimulation. In contrast, Poly(I:C), Z-VAD or LCL161 individual treatments had minor effects in inducing the expression of CXCL2 but significant levels of CXCL2 were detected in response to co-treatment of all 3 ligands. Taken together these results indicate necroptosis favours the expression of this chemokine and may contribute to the pro-inflammatory effects of necroptosis whilst also suppressing expression of the anti-inflammatory IL10.

5.3 Discussion

The use of proteasome inhibitors is one of the main approaches to treatment for diseases like MM. Bortezomib is one of these drugs, and it has been used for the past fifteen years. However, many patients do not respond to it or develop resistance to treatment. As part of this thesis, an increased expression of anti-inflammatory cytokine IL10 was observed when murine macrophages were treated with MG132, another proteasome inhibitor. IL10 expression and/or its polymorphisms have been broadly discussed and correlated to disease progression and poor prognosis in MM patients, both before and after treatment (Pappa *et al.*, 2007; Alexandrakis *et al.*, 2015; Freire-de-Lima *et al.*, 2017; Haydaroglu *et al.*, 2017; Kasamatsu *et al.*, 2017; Nielsen *et al.*, 2017). Some studies suggest IL10 acts as a growth factor for MM, and it has been shown before that this anti-inflammatory cytokine enhances monocytes differentiation when combined with macrophage colony-stimulating factor (M-CSF) *in vitro* (Hashimoto *et al.*, 1997; Pappa *et al.*, 2007; Kovacs, 2010; Deng *et al.*, 2012; Alexandrakis *et al.*, 2015). Therefore, an increase in this cytokine triggered by a proteasome inhibitor might suggest an unwanted secondary effect being induced by clinical treatment with proteasome inhibitors. Interestingly, our results showed an increase in IL10 expression induced by MG132 alone, comparable to the expression of this cytokine induced by LPS alone. It was expected that LPS would increase the expression of IL6, IL10, CXCL1 and CXCL2 due to its capacity to activate MyD88 and TRIF signalling pathways through TLR4 (Boonstra *et al.*, 2006; Iyer, Ghaffari and Cheng, 2010; Saraiva and O'Garra, 2010). The reduction in the expression of both cytokines and chemokines seen in presence of MG132 and LPS confirms that their induction was mediated by NF-κB, because blocking proteasome activity would interfere with degradation of its inhibitory protein IκBα and consequently the translocation of this transcription factor to the nucleus (Akira and Takeda, 2004). Interestingly, LPS induced expression of IL6 expression is completely abrogated with MG132 while IL10 expression remains at significant levels under the same conditions. In addition, activation of TLR4 has been shown to protect IL10 mRNA from degradation via TRIF and p38 signalling (Teixeira-Coelho *et al.*, 2014). The high expression of IL10 and considerably lower expression of IL6 in macrophages stimulated with TLR3 ligand Poly(I:C) (approximately 1000pg/mL and 400pg/mL respectively) could also be explained by type I IFNs signalling. These are consistent with findings suggesting macrophages are essential in regulating chronical viral infections through production of IL10 (Richter *et al.*, 2013).

In line with the results for cytokine expression by LPS, the chemokines CXCL1 and CXCL2 were also expected to be strongly increased considering their crucial role in inflammation (Wolpe *et al.*, 1989; Iida and Grotendorst, 1990; Schumacher *et al.*, 1992; Graham and Locati, 2013). The reduction of both chemokines with MG132 and LPS treatment was also anticipated, and it could be explained by the inhibition of IκBα degradation, as previously mentioned for IL6 and IL10. Considerable differences in the expression of both cytokines when comparing the effects of TLR4-ligand LPS or TLR3-ligand Poly(I:C) were also foreseen, given that bacterial-derived signals induce more robust inflammatory responses than the viral-derived ones (Akira & Takeda, 2004; O'Neill, Golenbock, & Bowie, 2013). Notably, we saw an increase in CXCL2 expression of approximately 2000pg/mL when macrophages were treated with MG132 alone. This was not seen for CXCL1. The differential effects of MG132 on the 2 chemokines are interesting considering CXCL2 shares approximately 90% homology with CXCL1 sequence (Iida and Grotendorst, 1990). However, CXCL2 has the capacity to attract polymorphonuclear leukocytes and hematopoietic stem cells, in addition to neutrophils (Wolpe *et al.*, 1989; Iida and Grotendorst, 1990). Interestingly, IL10 has been shown to downregulate CXCL2 in models of bacterial infection, viral infection, autoimmune disease and acute lung injury (Greenberger *et al.*, 1995; Kasama *et al.*, 1995; Tumpey *et al.*, 1998; Shanley, Vasi and Denenberg, 2000). Expression of CXCL8, another inflammatory chemokine closely related to CXCL2, was also reduced by IL10 in eosinophils stimulated with LPS (Takanaski *et al.*, 1994). Based on this evidence, IL10 and CXCL2 expression induced by MG132 seems to be independent of each other and it is more likely to be the result of signalling directly activated by the proteasome inhibitor. We evaluated the kinetics of upregulation of IL10 and found a considerable increase in IL10 at both mRNA and protein levels between 4 and 8 hours treatment with MG132. These data support MG132 directly triggering a signalling pathway which leads to the production of the anti-inflammatory cytokine. Furthermore, after 8 hours treatment with MG132, the upregulation of IL10 seems is much more marked. This could be explained by an autocrine response mediated by STAT3 signalling (Staples *et al.*, 2007).

Interestingly the positive effects of MG132 on IL10 expression appears to be specific to macrophages because MG132 failed to affect IL10 expression in dendritic cells or monocytes (THP-1). This may reflect findings in the literature that highlight macrophages and different subtypes of Th cells as the main sources of IL10 (Saraiva and O'Garra, 2010). Myeloid dendritic cells also produce IL10, but the present studies also show they are not responsive to MG132. It would be interesting to evaluate the production of IL10 induced by proteasome inhibition in the context of Th cells, and on MM cell lines. It could be also informative to evaluate co-cultures of

MM cells with macrophages to determine how IL10 production by the latter could alter proliferation of MM cells or if immunotolerance of macrophages can be induced by MM cells after treatment with proteasome inhibitors. An easy approach to study such interaction could be to culture MM cells in supernatants of macrophages treated with the different proteasome inhibitors, or vice versa, and subsequently assess changes in their proliferation, induction of cell death or functional activation. This may be of major relevance in a pathophysiological scenario and may have important consequences in the development of strategies to reverse resistance to treatment with proteasome inhibitors like Bortezomib in MM.

When looking at potential signalling pathways triggered by MG132 and capable of inducing IL10, the phosphorylation of P38, JNK and CREB in response to MG132 was especially noteworthy. MAPK P38 and CREB, have been shown to be crucial in induction of IL10 (Chi *et al.*, 2006; Kaiser *et al.*, 2009; Saraiva *et al.*, 2009; Wen, Sakamoto and Miller, 2010). Interestingly, ERK was dephosphorylated (also known as P44/42) and JNK was phosphorylated in macrophages treated with MG132 (Kaiser *et al.*, 2009; Saraiva and O'Garra, 2010). Moreover, when inhibitors for P38 and JNK were used, expression of both IL10 and CXCL2 was reduced almost to the level of non-treated cells. Considering P38 and JNK are usually described as the main MAP kinases involved in stress responses (Kyriakis and Avruch, 2012; Johnson and Lapadat, 2013), it is reasonable to expect their activation in response to accumulation of misfolded or damaged proteins in the cytoplasm as a result of proteasome inhibition. Also, as mentioned before, P38 is known to be important in IL10 production. Given the essential role of P38 and JNK pathways in central cellular processes like apoptosis, inflammation and cell proliferation and differentiation, they have also been strongly associated with proliferation of malignant cells where most of these pathways are usually altered (Wagner & Nebreda, 2009). P38 and JNK signalling pathways are key regulators keeping the balance between autophagy and apoptosis in response to genotoxic stress. For this reason, understanding and regulating their activation has recently been discussed as an approach for sensitizing cancer cells to chemotherapeutic agents (Sui *et al.*, 2014). In that sense, in a previous chapter of this thesis, it was shown that MG132 induces activation of apoptosis when in presence of LPS but not by itself (**Figure 4.15B**). Although, a slight increase in cleaved PARP is seen at 6 hours treatment with MG132 alone, the results suggest apoptosis is not triggered by the proteasome inhibitor alone. Experimental evidence with Bortezomib describes the phosphorylation of JNK and Bcl-2 as the pathways mediating autophagy in head and neck squamous cell carcinoma (HNSCC) cells (Li and Johnson, 2012). Furthermore, MG132 has been proven to sensitize TRAIL-resistant prostate cancer cells by activating c-Fos and c-Jun (Li, Zhang and Olumi, 2007). It is to note that both c-Fos and c-Jun

are part of the AP1 family, an important transcription factor in IL10 signalling (Ananieva *et al.*, 2008; Wen, Sakamoto and Miller, 2010). Based on these reports, proteasome inhibition by MG132 seems to have a strong effect on activation of multiple cascades related to IL10, but the main mechanism seems to be phosphorylation of MAPK P38.

The next step was to determine the effects of Bortezomib on IL10 expression. A clear increase in IL10 was apparent in macrophages treated with the clinically used proteasome inhibitor. Interestingly, our results contrast with a recent report by Chang and collaborators (2015) describing a decrease in immunosuppressive cytokines like IL10 and TGF-β1 in cutaneous T cell lymphoma (CTCL) cells treated with Bortezomib *in vitro*. The authors of the study suggested that the Bortezomib-dependant reduction of IL10 is independent of IκBα and connected to NF-κB non-canonical activation. They also showed inhibition of the chemokine receptor CXCR4 (a receptor from the CXC family like CXCL2) in CTCL cells as a result of Bortezomib treatment (Chang, Poltoratsky and Vancurova, 2015). In the same line, reduced expression of IL6 and IL10, as well as reduction in STAT3 phosphorylation was seen in mantle cell lymphoma cell lines after treatment with Bortezomib (Baran-Marszak *et al.*, 2010). In both of those cases, the effect of Bortezomib was characterised in cancer cells. However, Beyar-Kats and collaborators (2016) showed more recently that Bortezomib not only increases migration and proliferation of MM cells *in vitro* but also promotes pro-inflammatory macrophages and therefore disease progression. The same study found an increase in IL6 and IL10 in plasma samples of mice treated with Bortezomib (Beyar-Katz *et al.*, 2016). Interestingly a recent report has described a correlation between chemokine CCL27, its receptor CCR10 and IL10 expression as a potential mechanism mediating drug resistance to proteasome inhibition in MM. The researchers described this effect to be dependent on the interaction between myeloma cells and stromal cells, and they succeeded in reversing drug resistance by blocking CCR10, IL10 or IL10R (Thangavadivel *et al.*, 2016). Our results suggest that IL10 expression induced by Bortezomib is also dependant on P38 and CREB activation but independent of ERK, as previously seen with MG132 treatment. However, the phosphorylation state of JNK seems to be differentially induced by the two proteasomal inhibitor evaluated. While MG132 induced phosphorylation of JNK, this effect was not as obvious with Bortezomib. Such observations were confirmed with the inhibitors for P38 and JNK, where inhibition of P38 depleted Bortezomib-induced expression of IL10 but inhibition of JNK did not affect it. The present evidence clearly indicates an association between IL10 expression in macrophages and the use of proteasome inhibitors. This may be of particular relevance to the treatment of MM since whereas Bortezomib may be able to suppress IL10 in tumour cells it may have the opposite effect in

stromal cells such as macrophages, as demonstrated in the present study. This may lead to patients becoming refractory to treatment with Bortezomib.

The expression of IL10 in different types of cell death was also evaluated since its expression was coincident with cytotoxicity and the main mechanism of action of Bortezomib is to induce or sensitize malignant cells to cell death. Bortezomib caused cytotoxicity in macrophages as revealed by the release of LDH into cell supernatants. However, LDH is not a specific signature of a particular form of cell death. Therefore, three types of program cell death were evaluated as potential triggers of IL10 expression/secretion. The results suggest the anti-inflammatory cytokine is not being induced by pyroptosis or necroptosis. These results are consistent with the well described pro-inflammatory nature of both types of cell death (Galluzzi *et al.*, 2014; Kolb *et al.*, 2017). As for apoptosis, our results show a modest increase in IL10 expression in response to cell death activated by TLR4 signalling and a significant reduction in the case of TLR3 signalling. These seems to be in line with the anti-inflammatory nature of apoptosis (Kolb *et al.*, 2017). In that sense, multiple studies report reduction or prevention of apoptosis correlated to increase on IL10 expression or treatment with the cytokine in many different models, but mainly in Treg and endothelial cells (Pan *et al.*, 2013; Yin *et al.*, 2013; Li *et al.*, 2014; Lao *et al.*, 2015; Behrendt *et al.*, 2016). In the specific case of treatment with Bortezomib, apoptosis has been shown to increase while IL10 is reduced (Baran-Marszak *et al.*, 2010).

Clearly, genetic variability and polymorphisms of IL10 have an important role in MMs poor prognosis and progression, but also the effect of treatment on specific cells and their interaction in the context of cancer microenvironment needs to be discussed. In that sense, macrophages have been shown to change their phenotypes in cancer development leaning towards an immunosuppressive type, and more likely to sustain malignant progression and metastasis (Biswas, Sica and Lewis, 2008). Another point to be considered in our approach is the specific mechanism by which MG132 and Bortezomib exert their function and the effect it has on subsequent changes in the cells. MG132 has been described as a weaker inhibitor of hydrolytic caspase and chymotrypsin-like catalytic activity compared to Bortezomib. However, both were reported to sensitize MM cells to apoptosis *in vitro* (Crawford *et al.*, 2006). It would also be of interest to assess the effect on IL10 production of irreversible binding proteasome inhibitors such as Carfilzomib, Marizomib or Oprozomib in the macrophages, considering the reduction in phosphorylation of P38 and CREB seen with the reversible binding proteasome inhibitor Bortezomib after 3 hours treatment (Teicher and Tomaszewski, 2015).

In summary, the results of this Chapter describe the induction of IL10 expression in macrophages by the proteasome inhibitor MG1323 and the clinically used Bortezomib. It is possible that IL10 expression is induced by stress signals that are triggered by protein accumulation in the cytosol. Phosphorylation of P38 and JNK, two well-known MAPKs activated by stress signals, seems to be the proximal mechanisms being activated under these conditions (**Figure 5.16**). Increase phosphorylation of P38 mediates the activation of transcription factor CREB, which in turn induces expression of IL10 and CXCL2. These two MAPKs can then act as growth factors or promotors of tumoural progression and IL10 at the same time could be inhibiting the immune response against the malignant cells. It was also shown that inhibition of P38 kinase activity by means of AMG548 stops MG132 and Bortezomib-induced IL10 (**Figure 5.16**). Furthermore, IL10 induction is not an intrinsic feature of pyroptosis, necroptosis or apoptosis. It is important to note that although the effects of the two proteasome inhibitors described in this thesis (MG132 and Bortezomib) were mostly similar in terms of inducing expression of IL10 and activation of P38 and CREB signalling, their impact in early signalling activation were to some extent different. Therefore, the efficiency and specificity of their responses needs to be considered in further experiments. Despite the strong correlation between the two mentioned proteasome inhibitors and the induction of anti-inflammatory cytokine IL10, further studies are required to determine the specificity of this response and to fully understand the underlying mechanism. It would be interesting to evaluate if similar observations are seen in MM cells or tumour-associated macrophages (TAM), or if other types of cells in the bone marrow microenvironment are also involved. A more comprehensive study on the effect of proteasome inhibition in cellular interactions and induction of soluble factors like IL10 and CXCL2 would be extremely useful to increase the efficiency of this type of treatment. This is especially relevant and timely in the light of the new generation of proteasome inhibitors awaiting to be approved for MM treatment.

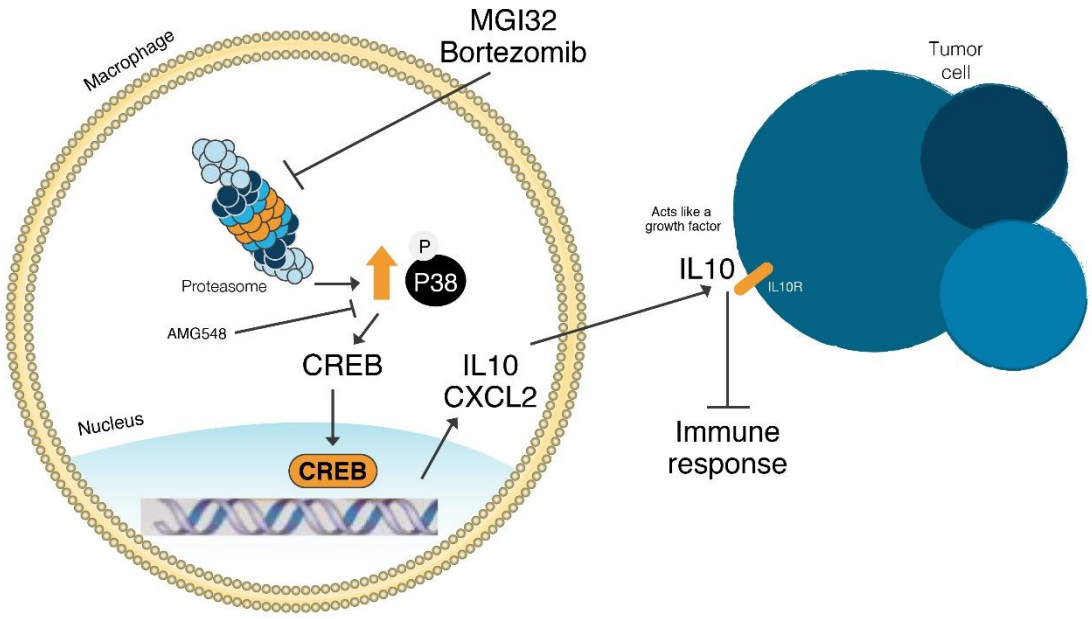

Figure 5.16 Model of IL10 production induced by proteasome inhibitors MG132 and Bortezomib.

Proteasome inhibition with MG1323 or Bortezomib induces the phosphorylation of P38 and to some extend JNK (specifically for treatment with MG132), two well-known MAPKs activated by stress signals. P38 in turn activates transcription factor CREB that will mediate the production of IL and CXCL2. When released in the tumoural microenvironment, IL10 can promote tumoural progression acting as a growth factor and inhibit further immune response against the malignant cells. Inhibition of P38 signalling cascade with AMG548 was showed to block the expression of IL10 induced by MG132 and Bortezomib.

CHAPTER SIX
CONCLUDING REMARKS

6.1 Concluding remarks

The present work aimed to explore elements of the ubiquitination and proteasome pathways in the context of cell death and innate immune signalling, especially in macrophages. Initial studies evaluated whether Pellino 2 and Pellino 3 E3 ubiquitin ligases play a role in the regulation of necroptosis by virtue of their close molecular and functional relationships with RIP kinases. The data show that neither E3 ligase regulates necroptosis. This contrasts with emerging roles for the remaining family member Pellino 1 in regulating necroptosis. However, this is complicated by two recent reports describing opposite roles for Pellino 1 in necroptosis with its definitive function in this pathway remaining to be clarified (Wang *et al.*, 2017; Choi *et al.*, 2018). It is also interesting to note that overexpression of Pellino drives tumour formation, especially lymphomogenesis and raising the possibility that necroptosis may be a novel mechanism to control tumour cells (Park *et al.*, 2014). The results also highlight the functional variability between the members of the Pellino family and promotes further investigation into their molecular and functional roles.

Another pathway that is strongly regulated by the ubiquitination / proteasome system is the transcription factor HIF-1α and the present studies show that all tested TLRs can promote stabilisation of HIF-1α. This is consistent with previous reports on the close functional relationship of HIF-1α with other transcription factors like NF-κB that are also activated by TLRs. Thus, whilst HIF-1α has been traditionally studied in the context of responding to hypoxia, it is also likely to play a key role in innate immunity. Furthermore, the findings also highlight that whilst HIF-1α is known to upregulate the enzymes in the glycolytic pathway, blockade of glycolysis suppresses the levels of HIF-1α suggesting a positive regulatory loop system that would facilitate amplification of signals in a short time frame. Furthermore, whilst both TLRs and hypoxia stabilise HIF-1α, TLRs appears capable of also stabilising a uniquely smaller form of HIF-1α. It was not possible to functionally characterise this latter form, but mass spectrometry analysis may be useful in determining its identity. This would provide important insight into its genesis and may offer clues to its functional relevance. In hypothesising that it may represent a processed form of full length HIF-1α, we explored the role of the proteasome as a mediator of this putative processing event. In doing so, the studies serendipitously highlighted that proteasome blockade strongly induced IL10 in macrophages. Whilst these conditions are also associated with cell death, the expression of IL-10 appears to be functionally separate from various forms of cell death. The importance of IL-10 expression in response to treatment of macrophages with proteasome inhibitors is discussed in the context of Bortezomib as a

therapeutic strategy in the treatment of multiple myeloma. The induction of IL-10 in macrophages may represent a valuable insight into the refractory nature of MM in the context of Bortezomib treatment. It would be fascinating to examine IL-10 in the different cell populations of bone marrow aspirates from MM patients undergoing Bortezomib treatment to evaluate if it displays opposing roles on IL-10 expression in lymphoma cells and resident stromal macrophages.

Overall this work emphasises the importance of the ubiquitination and proteasome system for cell death and innate immunity. It offers some new insights into these relationships and prompts further studies that may have applications that extend from understanding molecular mechanisms of inflammatory disease or the treatment of cancer.

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