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INFLAMMATORY CONSEQUENCES OF DIVERGENT CELL DEATH SIGNALS

Conor J. Kearney

Thesis submitted to Trinity College Dublin for the degree of Doctor of Philosophy.

Thesis supervisor: Prof. Seamus J. Martin

Molecular Cell Biology Laboratory,

Department of Genetics,

University of Dublin,

Trinity College,

Dublin 2

DECLARATION

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SUMMARY

Tumor Necrosis Factor (TNF) is an apical cytokine that drives inflammation through triggering the synthesis and secretion of multiple downstream pro-inflammatory cytokines and chemokines. Generally, TNF stimulation does not result in cell death, however, under particular conditions, TNF can drive apoptosis or necroptosis. The Inhibitor of Apoptosis Proteins (IAPs) often dictate whether TNF stimulation results in cytokine secretion and cell survival, or alternatively, cell death. This occurs by facilitating NFκB activation through ubiquitination of RIPK1. Furthermore, TNF can induce a form of regulated necrosis, called necroptosis if caspase-8 activity is inhibited. Necroptosis is also inhibited by IAPs and is thought to be initiated by RIPK1 and RIPK3. Indeed, necroptosis represents an alternative and unusual outcome of TNF stimulation, preventing normal TNF function in driving cytokine and chemokine production, but promoting release of Danger Associated Molecular Patterns (DAMPs).

CHAPTER 3

IAPs are known to play a major role in determining whether cells undergo apoptosis in response to TNF as well as other stimuli. This realization was brought about by the development of small molecule antagonists of IAPs, called smac mimetics. Indeed, IAP antagonists dramatically sensitise transformed cells to TNF-induced apoptosis in a RIPK1-dependent manner. However, TNF is also highly pro-inflammatory through its ability to trigger the secretion of multiple inflammatory cytokines and chemokines, which is arguably the most important role of TNF *in vivo*. Indeed, deregulated production of TNF-induced cytokines is a major driver of inflammation in several autoimmune conditions such as rheumatoid arthritis. As IAPs are thought to facilitate TNF-induced signaling pathways, including NFκB activation, we explored the contribution of IAPs to the production of TNF-induced pro-inflammatory cytokines. Indeed, we found that IAPs are required for optimal production of multiple TNF-induced pro-inflammatory mediators. Ablation of IAPs potently suppressed TNF- or RIPK1-induced pro-inflammatory cytokine and chemokine production, independent of sensitization towards apoptosis. Interestingly, IAP antagonism also led to spontaneous production of chemokines,

particularly RANTES, *in vitro* and *in vivo* which may have implications for the use of these molecules as cancer therapies. Thus, IAPs play a major role in influencing the production of multiple inflammatory mediators, arguing that these proteins are important regulators of inflammation in addition to apoptosis.

CHAPTER 4

Necrosis is thought to represent an inflammatory cell death mode, due to release of intracellular constituents called Damage Associated Molecular Patterns (DAMPs), that can activate the innate immune system. While necrosis often occurs as a result of physical injury or overwhelming toxic agents, in special circumstances necrosis can also proceed in a programmed and controlled fashion called necroptosis. Because necroptosis results in rapid membrane rupture, it is widely presumed that TNF-induced necroptosis is detrimental *in vivo* due to DAMP release, driving excessive inflammation. However, because TNF is intrinsically highly pro-inflammatory, rapid cell death via necroptosis may blunt rather than enhance TNF-induced inflammation. Thus, the relative inflammatory potential of TNF-induced cytokines/chemokines compared to DAMP release is unknown in this context. We have explored this issue, finding that TNF-induced necroptosis potently suppressed the production of multiple TNF-induced cytokines and chemokines due to RIPK3-dependent cell death. Similarly, necroptosis also suppressed LPS-induced pro-inflammatory cytokine and chemokine production. Knockdown of RIPK3 under necroptosis-inducing conditions blocked cell death and restored TNF-driven cytokine production. Importantly, supernatants from TNF-stimulated cells were substantially more pro-inflammatory than those from TNF-induced necroptotic cells in vivo. Thus, necroptosis attenuates TNF-induced inflammatory responses, which is not compensated for by the release of DAMPs. We therefore suggest that intracellular pathogens may evoke necroptosis to terminate host inflammatory responses.

CHAPTER 5

Ligands of the TNF superfamily and Toll-Like Receptor (TLR) family such as LPS and PolyIC, can promote a regulated form of necrosis, called necroptosis, upon inhibition of caspase activity in cells that express RIPK3. Cell death is often defined as necroptosis if it is prevented by Necrostatin-1, a potent and specific inhibitor of RIPK1 kinase activity. Because Necrostatin-1 blocks necroptosis in all settings, RIPK1 kinase activity is thought to be required for activation of RIPK3, leading to necroptosis. However, we have found that although Necrostatin-1 potently inhibited TNF- or toll ligand induced necroptosis, knockdown of RIPK1 dramatically potentiated this process. Importantly, Necrostatin-1 failed to block necroptosis in the absence of RIPK1, indicating that this inhibitor was acting in an on-target fashion. Therefore, we argue that RIPK1 is dispensable for necroptosis and indeed can act as an inhibitor of this process. Our observations also suggest that Necrostatin-1 works through enhancing the inhibitory properties of RIPK1 on necroptosis, rather than blocking its participation in this process. Taken together, this study demonstrates that the current model of RIPK1/RIPK3- dependent necroptosis should be re-evaluated, and provides an unexpected insight into how Necrostatin-1 blocks necroptotic cell death.

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ABBREVIATIONS

ADV	Adenovirus
ATP	Adenosine tri-phosphate
AxV	Annexin V
APAF	Apoptotic protease activation factor
CARD	Caspase activation and recruitment domain

cIAP	Cellular inhibitor of apoptosis protein
cFLIP	Cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
CIRP	cold-inducible RNA-binding protein
CLP	Cecal ligation and puncture
CMV	Ctyomegalovirus
CrmA	Cytokine response modifier A
CXCL1	C-X-C motif ligand 1
CYLD	Cylindromatosis
DAMP	Danger- associated molecular pattern
dATP	Deoxyadenosine triphosphate
ddH_20	Double distilled H_20
DED	Death effector domain
DISC	Death- inducing signaling complex
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenadiaminetetraacetic acid
ERK	Extracellular regulated kinase
FACS	Fluorescence-activated cell sorter
FADD	Fas- associated death domain
Fas	First apoptosis signal
FasL	Fas ligand
FITC	Flourscin isothiocyanate

FMK	Fluoreomethylketone
GFP	Green fluorescent protein
GMCSF	Granulocyte-macrophage colony-stimulating factor
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HMGB1	High-mobility group protein B1
HOIL-1	Heme-oxidized IRP2 ubiquitin ligase 1
HOIP	HOIL-1- interacting protein
IAP	Inhibitor of apoptosis protein
IKBα	NF-kappa-B inhibitor alpha
IKK1	IkappaB kinase 1
IKK2	IkappaB kinase 2
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
Il-33	Interleukin-33
IRF-3	Interferon regulatory factor 3
JNK	c-Jun N-terminal kinase
KC	Keratinocyte- derived cytokine
kDa	Kilodaltons
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin chain assembly complex

MEF	Mouse embryonic fibroblast
MEK	Mitogen-activated protein kinase kinase
MKK3	Mitogen-activated protein kinase kinase kinase 3
MKK6	Mitogen-activated protein kinase kinase kinase 6
MLKL	Mixed lineage kinase domain-like
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
NIK	NFκB-inducing kinase
Oligo	Oligonucleotide
P38	p38 mitogen activated protein kinase
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen- associated molecular pattern
Poly IC	Polyinosinic:polycytidylic acid
PBS	Phosphate buffered saline
PIPES	Piperazine-N-N'-bis(2-ethanesulphonic acid)
RANTES	regulated on activation, normal T cell expressed and secreted
RHIM	RIP homotypic interaction motif
RIG-I	Retinoic acid-inducible gene 1
RING	Really interesting new gene
RIPK1	Receptor- interacting Serine/Threonine protein kinase 1
RIPK3	Receptor- interacting Serine/Threonine protein kinase 3
ROS	Reactive oxygen species

RPMI	Roswell Park Memorial Institute
siRNA	Small interfering RNA
SIRS	Systemic inflammatory response syndrome
TAE	Tris acetate EDTA
TAB-1	TGF-beta activated kinase 1- binding protein 1
TAB-2	TGF-beta activated kinase 1- binding protein 2
TAK-1	TGF-beta activated kinase 1
TB	Tuberculosis
TE	Tris EDTA
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-1- associated death domain protein
TRAF2	TNF receptor-associated factor 2
TRAF3	TNF receptor-associated factor 3
TRAIL	Tumor necrosis factor- related apoptosis- inducing ligand
TRIF	TIR domain-containing adaptor protein inducing interferon β
TRPM7	Transient receptor potential cation channel, subfamily M, member 7
UV	Ultraviolet radiation
XIAP	X-linked inhibitor of apoptotic proteases
zVAD-FMK	Benzylocarbonyl-Val-Ala-Asp-(OMe) fluoromethylketone

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CHAPTER 1

INTRODUCTION

1.1 OVERVIEW OF CELL DEATH

Cell death is a naturally occurring process in the body. In fact, billions of our cells die every day, and this is required for normal bodily function. Cell death has been intensively studied over the past 25 years and distinct forms of cell death have been identified. Two major modes of cell death include apoptosis and necrosis. Apoptotic and necrotic forms of cell death are distinguishable by morphological appearance, enzymatic involvement and immunological consequence (Davidovich et al., 2014; Henry et al., 2013; Taylor et al., 2008).

1.1.1 Introduction to apoptosis

Apoptosis is a form of programmed cell death in which cells are dismantled from within, a process that minimizes damage to surrounding cells (Martin et al., 2012). The signaling pathways, genetic control and final execution of the apoptotic program has been well documented (Taylor et al., 2008). Apoptosis is essential to many vital processes including the maintenance of cell proliferation and cell death homeostasis (Danial and Korsmeyer, 2004), removal of T-cells that possess autoimmune potential (Werlen et al., 2003) and body shape formation during development (Jacobson, 1997). Apoptosis is morphologically characterised by cell shrinkage, membrane blebbing, chromatin condensation, and formation of apoptotic bodies. During apoptosis, cysteine proteases called caspases, dismantle cells in an organized and programmed nature, packaging cell constituents in a safe fashion for phagocytic engulfment. This process ensures that apoptosis does not result in explicit activation of the immune system. Apoptosis may be initiated by extracellular cytokines, particularly members of the Tumor Necrosis Factor (TNF) superfamily, known as the extrinsic pathway (Ashkenazi and Dixit, 1998). This pathway activates caspase-8 at receptor complexes, leading to cellular demise through proteolytic cleavage of executioner caspases-3 and -7 (Figure 1.1). Alternatively, apoptosis can be initiated internally, as a result of cell damage or stress. This is known as the intrinsic pathway. This pathway is activated via cytochrome release from mitochondria, leading to Caspase-9 activation in the apoptosome, and subsequent proteolytic cleavage of caspases-3 and -7 (Slee et al., 1999; Taylor et al., 2008) (Figure 1.1)

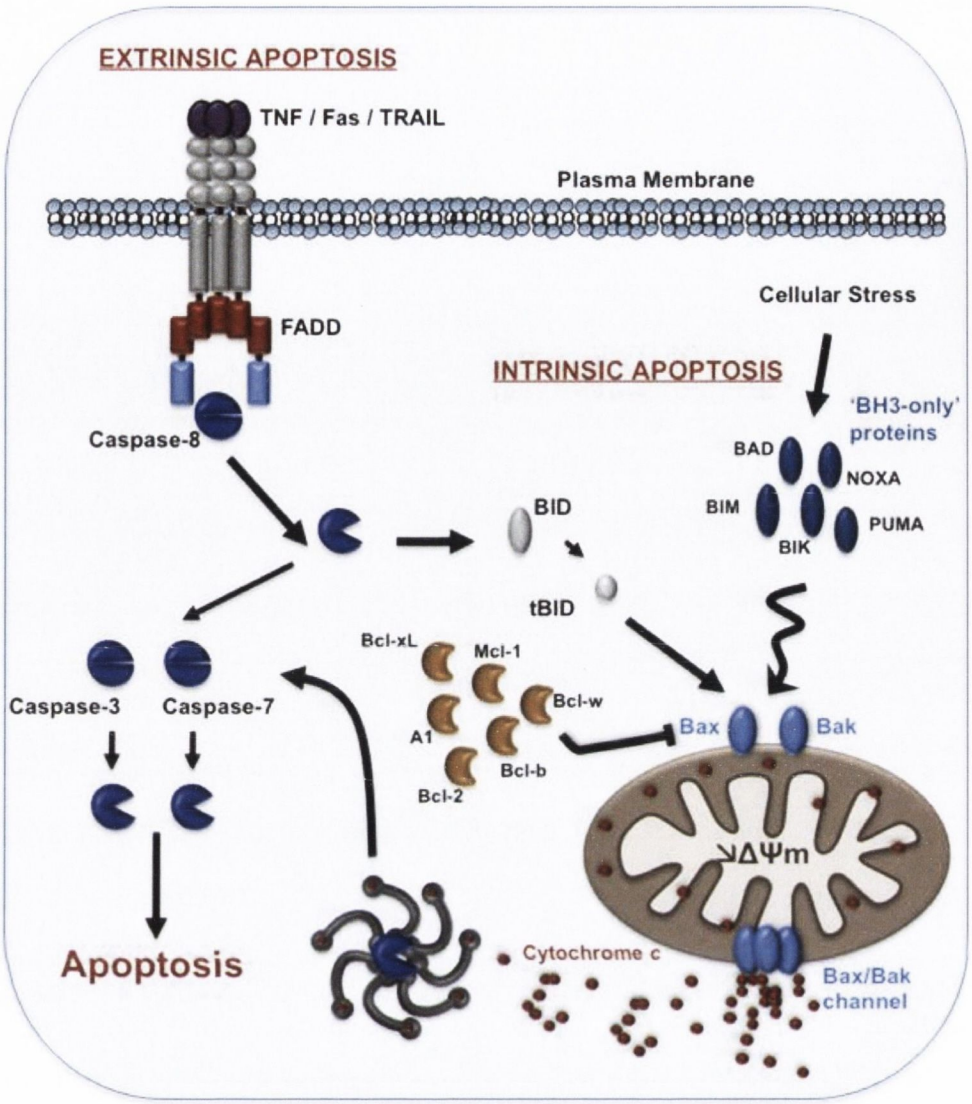

Figure 1.1

The extrinsic and intrinsic apoptosis pathways

The extrinsic pathway to apoptosis can be initiated by members of the TNF superfamily including TNF, Fas and TRAIL, that bind to trans-membrane 'death receptors'. Caspase-8 molecules are then recruited to FADD, where they become activated by proximity-induced auto-activation. Caspase-8 then proteolytically cleaves executioner caspases- 3 and -7, leading to apoptosis. Caspase-8 can also cleave BID, generating a tBID molecule that transfers to the mitochondria to drive the intrinsic apoptosis pathway, as now discussed. The intrinsic pathway to apoptosis is activated when cytochrome c is released from mitochondria. Cytochrome c then activates caspase-9 in the apoptosome, leading to activation of caspases-3 and -7 and apoptosis. BH3-only proteins respond to cell stress by activating Bax and Bak, which serve as cytochrome c release channels. This process is negatively regulated by the anti-apoptotic Bcl-2 members. BH3 proteins can also indirectly activate Bax and Bak by binding and neutralizing anti-apoptotic Bcl-2 members.

1.1.2 Introduction to necrosis

Necrosis has long been considered an accidental form of cell death, caused by sudden physical insult, deregulated osmotic pressure, or exposure to toxic stimuli such as H_2O_2. Necrosis results in catastrophic release of cell constituents into the extracellular space, often damaging surrounding tissue. As necrosis is a sign of danger, injury or infection it results in activation of the immune system and subsequent inflammation. In contrast to apoptosis, necrosis has long been thought to lack any level of molecular control (Abraham and Shaham, 2004; Kroemer and Martin, 2005). This view has changed in recent years with the identification of controlled signaling networks that culminate in a necrotic-like cell death called necroptosis (Kaczmarek et al., 2013). Necroptosis is often initiated by members of the TNF superfamily, similarly to apoptosis. A defining feature of necroptosis, however, that distinguishes it from apoptosis, is that it proceeds only when caspase activity is inhibited.

1.2 OVERVIEW OF INFLAMMATION

Inflammation is a complex set of reactions that occurs in response to infection and tissue injury (Medzhitov, 2008). We have all experienced common symptoms of inflammation; swelling, pain, redness and increased body temperature. Inflammation is triggered by cells of our innate immune system, often macrophages or dendritic cells that reside in all tissues in anticipation of infection or damage. Sensing of danger by innate immune cells is mediated by pattern recognition receptors (PRRs), which recognize molecular signatures from invading pathogens or from endogenous molecules (Danger Associated Molecular Patterns, DAMPs) that are released as a result of necrotic tissue damage. Upon PRR activation, these cells initiate a transcriptional program, leading to release of cytokines such as TNF and Interleukin-1 (IL-1). These apical cytokines promote the involvement of multiple other immune and non-immune cell types, thus amplifying inflammation. Indeed, infection and injury usually coincide with cell death, thus cell death and inflammation are related biological events with one often leading to the other (Matzinger, 1994). Furthermore, certain molecules that promote inflammation, particularly TNF, can often also promote cell death, be it apoptosis or necrosis.

1.2.1 Cytokines and chemokines drive innate immunity and inflammation

Cytokines are a diverse family of small secreted proteins (5-20 kDa) that have a specific effect on the interactions and communication between cells, often innate immune cells (Dinarello, 2007). Cytokines include TNF family ligands, chemokines, interleukins, interferons, and lymphokines. Cytokines may act on the cell they are secreted from (autocrine), on neighboring cells (paracrine) or on peripheral cells or distant cells (endocrine). Cytokines usually operate in a cascade effect, whereby an apical cytokine stimulates the production of other cytokines from responding cells. In this regard, TNF and IL-1 may be considered the predominant apical cytokines as they can stimulate production of virtually all other cytokines, thus shaping immune responses. As macrophages are rich sources of TNF and IL1, they are often the cells that initiate inflammation. Chemokines are a subset of cytokines (chemotactic cytokines) that promote chemotaxis of leukocytes. Over 30 human chemokines exist and fall into two major groups, CXC chemokines and CC chemokines, referring to the two conserved N-terminal cysteines (Zlotnik and Yoshie, 2012). A third group with a solitary member, fractalkine/CX3CL1, which is a potent monocyte and T-cell attractant, adopts a unique CX3C motif (Truman et al., 2008) (Table 1).

Tissue inflammation is typified by neutrophil infiltration. Chemokines such as IL-8, CXCL1/KC, RANTES, and MIP-2 are potent neutrophil chemoattractants (Charo and Ransohoff, 2006). Thus, a TNF-induced gradient of these chemokines facilitates neutrophil migration into peripheral tissues where they deploy aggressive antimicrobial mechanisms. MCP-1 is a monocyte chemotactic chemokine. MCP-1 facilitates monocyte and macrophage migration into inflamed tissue and is also released by apoptotic cells to aid their clearance by phagocytes (Charo and Ransohoff, 2006; Cullen et al., 2013). RANTES is a chemokine that guides $CD4^+$ T-cells into inflamed sites or Naive T-cells into the proximity of antigen presenting cells (APCs) to facilitate rapid clonal expansion. Cytokines that have more systemic effects include IL-6 and GMCSF. IL-6 is a signature of acute phase immunity and is rapidly synthesized following TNF stimulation. IL-6 is capable of crossing the blood brain barrier to influence the hypothalamus thermostat, increasing body temperature to slow pathogen replication. It also systemically travels to the liver, facilitating complement synthesis and release, molecules that are highly efficient at

Cytokine	Class	Apical Cytokine	TNF-Inducible	Predominant Responder Type	DAMP Properties
TNF	Cytokine	Yes	Yes	Most cells	No
IL1-β	Interleukin	Yes	Occasionally	Most cells	Yes
IL1-α	Interleukin	Yes	Occasionally	Most cells	Yes
IL-33	Interleukin	Yes	Occasionally	Most cells	Yes
IL-6	Cytokine	Yes	Yes	Epithelium, Hypothalamus, Liver	No
GMCSF	Cytokine	No	Yes	Hematopoietic progenitors	No
MIP-2/ CXCL2	Chemokine CXC	No	Yes	Neutrophils	No
IL-8/ CXCL8	Chemokine CXC	No	Yes	Neutrophils	No
KC/ CXCL1	Chemokine CXC	No	Yes	Neutrophils	No
MCP-1	Chemokine CC	No	Yes	Monocytes, Macrophages	No
RANTES/ CCL5	Chemokine CC	No	Yes	T-cells, Neutrophils	No

Table 1.

Classification and function of common cytokines and chemokines

lysing bacteria (Hibi et al., 1996). Epithelial and endothelial cells specialize in GM-CSF release upon TNF stimulation. GM-CSF stimulates a dramatic increase in hematopoietic progenitor cells to differentiate into effector granulocytes, mainly neutrophils, basophils and eosinophils to join the immune response in greater numbers (Hamilton and Anderson, 2004). It can be appreciated, therefore, that apical cytokines, including TNF, instigate an inflammatory cascade involving many other cytokines and chemokines that orchestrates innate immunity.

Most conventional cytokines and chemokines contain secretory leader sequences, thus they are trafficked from the ER to the plasma membrane where they are released into the extracellular space. Particular cytokines, however, such as members of the IL1 family (IL-1β, IL-1α, IL-33, IL-36) do not possess such sequences and are therefore sequestered within viable cells. As these cytokines are liberated during necrotic cell death and act as apical cytokines, they serve as potent DAMPs. A summary of cytokine and chemokine function is provided in Table 1.

1.3 TUMOR NECROSIS FACTOR (TNF)

1.3.1 TNF discovery and structure

During the late 1960s, two independent reports emerged describing a cytotoxic factor released from lymphocytes, which was named lymphotoxin (Kolb and Granger, 1968; Ruddle and Waksman, 1968). Several years later, a bacterial endotoxin-induced factor derived from macrophages was shown to induce necrosis of mouse fibrosarcoma L929 cells, which was named tumor necrosis factor (TNF) (Carswell et al., 1975). The genes encoding TNF and lymphotoxin were later cloned in 1984, revealing sequence similarity. It was soon realized that a secreted hormone from cultured RAW macrophages, called cachectin, that was known to modulate metabolism, was actually TNF (Beutler et al., 1985). As a result, TNF was later renamed TNF-α while lymphotoxin was renamed TNF-β.

TNF exerts many of its biological effects through binding as a 52 kDa trimer to one of two receptors, TNF-R1 and TNF-R2 (Black et al., 1997) (Loetscher et al., 1993;

Smith and Baglioni, 1987). TNF is produced as a type II trans-membrane protein arranged in stable homotrimers. Membrane bound TNF can then be proteolytically cleaved by the metalloprotease TNF alpha converting enzyme (TACE), to shed a soluble 17 kDa active signaling protein (Black et al., 1997; Mohan et al., 2002). Indeed, membrane bound TNF is believed to have different signaling capabilities from that of soluble TNF, as does monomeric versus trimeric soluble TNF. This aspect of TNF biology, however, remains poorly understood (Ardestani et al., 2013; Ruuls et al., 2001). Each TNF monomer contains two antiparallel β-sheets with antiparallel β-strands, forming a 'jelly-roll' structure (Eck and Sprang, 1989).

1.3.2 TNF Function

Members of the TNF super family including TNF, Fas and TRAIL promote diverse cellular outcomes upon binding to their associated receptors. Fas and TRAIL are traditionally considered death-inducing molecules, initiating apoptosis through the extrinsic pathway, as described previously (Figure 1.1). Despite the context in which TNF was discovered, extensive research has revealed that the main function of TNF lies with immunity and inflammation, not cell death. However, TNF does have the ability to kill cells under particular conditions, which are now relatively well understood (Wajant et al., 2003). TNF can induce apoptosis if the pro-survival effects of the Nuclear Factor Kappa B (NFκB) transcription factors are blocked (Van Antwerp et al., 1996). Furthermore, TNF can also induce necroptosis if capsase-8 activity is blocked (He et al., 2009; Lin et al., 2004; Mocarski et al., 2012) (Figure 1.2). However, under most physiological circumstances, in the absence of pharmacological intervention or genetic alterations, TNF promotes inflammation without killing the cell it engages (Figure 1.3). Naturally, TNF is an extremely inflammatory molecule and is instrumental in innate immunity. Upon TNF stimulation, cells mount a robust transcriptional program through activation of NFκB transcription factors, leading to secretion of downstream cytokines that have a critical function in shaping and directing the immune response, as mentioned above. In this regard, perhaps the most important TNF target genes are a battery of other cytokines and chemokines (Figure 1.3). A second subset of molecules are transcriptional targets of TNF activity through NFκB activation. These molecules,

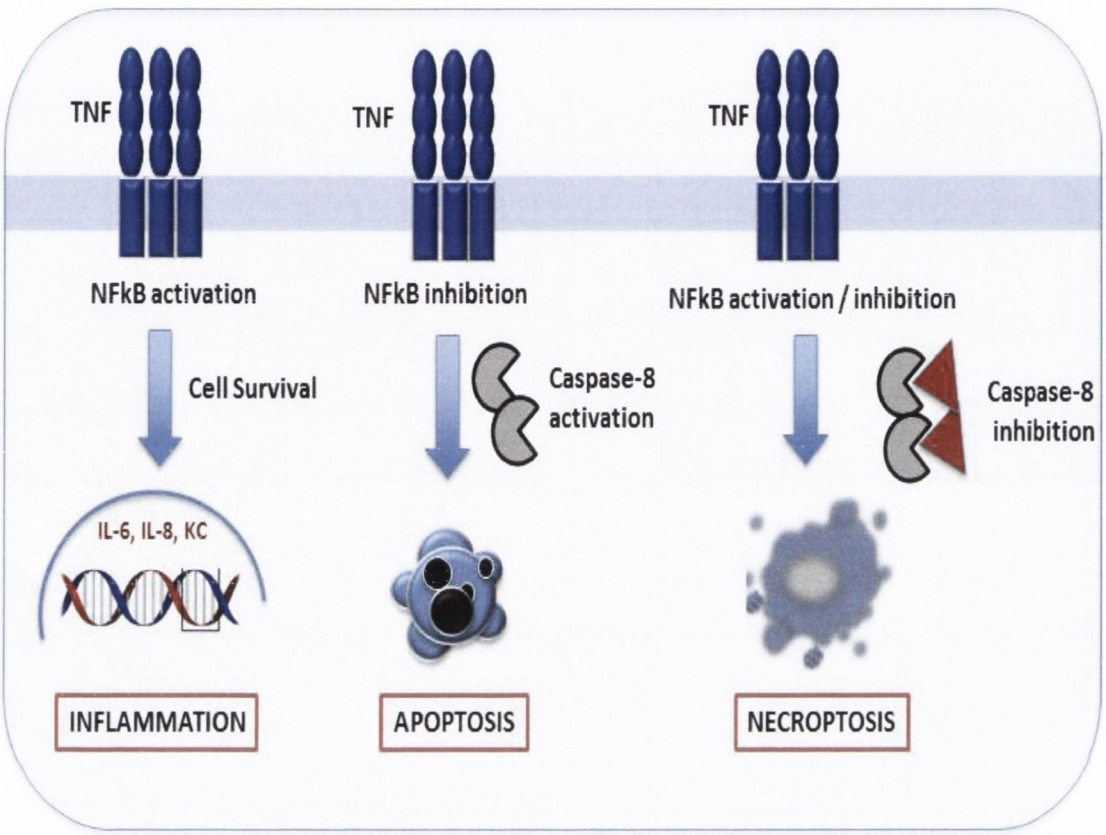

Figure 1.2

Diverse outcomes of TNF signaling

TNF stimulation of most cell types alone does not result in cell death but triggers NFκB activation which drives synthesis and secretion of multiple pro-inflammatory cytokines and chemokines. If activation of NFκB transcription factors is inhibited, however, TNF stimulation can lead to activation of caspase-8 and subsequent apoptosis. Furthermore, if caspase-8 activity is inhibited. TNF stimulation can drive a regulated form of necrosis called necroptosis. In some cell types, caspase-8 inhibition alone is sufficient to drive TNF-induced necroptosis while other cell types require combined inhibition of NFκB activation and caspase-8 activity to initiate necroptosis.

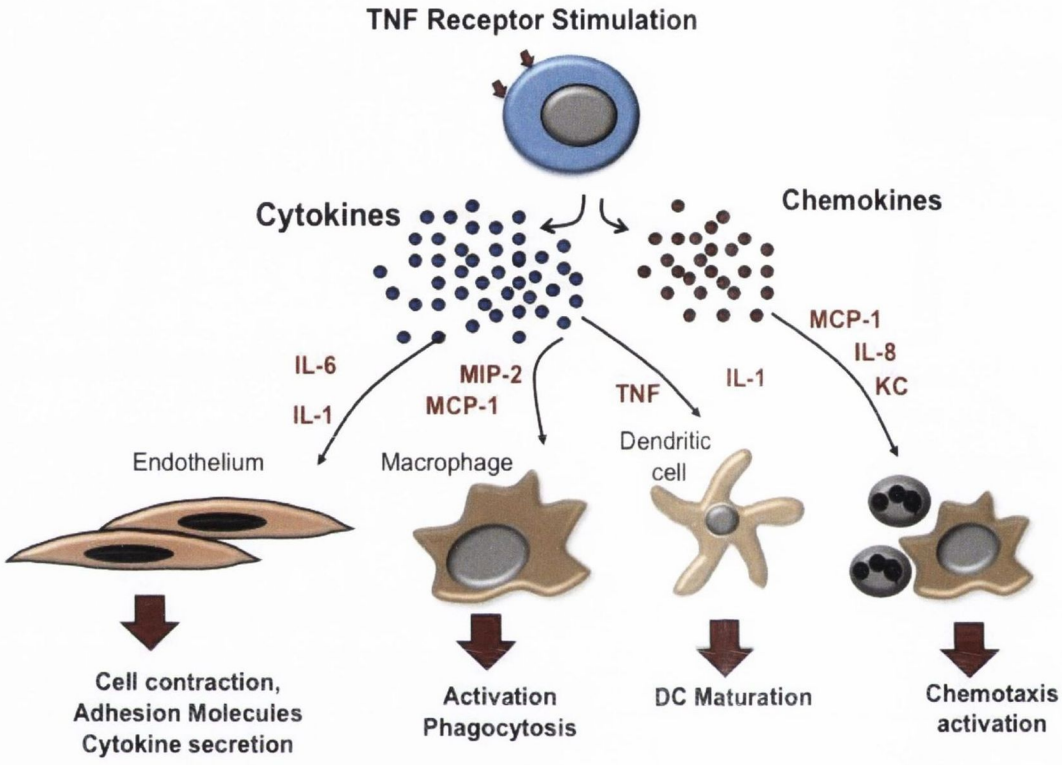

Figure 1.3

TNF engages multiple arms of the immune system by triggering secretion of cytokines and chemokines

Multiple cell types respond to TNF by secreting a battery of cytokines and chemokines. IL-6 and IL-1 upregulate adhesion molecules on local endothelium, facilitating neutrophil migration from blood into tissue. MIP-2 and MCP-1 enhance the phagocytic activity of macrophages while TNF and IL1 can directly promote dendritic cell maturation. TNF- induced chemokines such as KC and MCP-1 promote chemotaxis of innate immune cells into inflamed tissue.

which include the cellular inhibitor of apoptosis proteins (cIAPs) and cFLIP promote cell survival by acting in a positive feedback loop to ensure the inflammatory signal is prolonged and not converted to an apoptotic signal via caspase-8 recruitment to the TNF receptor (Panayotova-Dimitrova et al., 2013; Varfolomeev et al., 2007; Vince et al., 2007; Wang et al., 2008).

TNF is predominantly produced by activated macrophages, however, it can also be produced by neutrophils, mast cells, endothelial cells, lymphocytes and even neurons. As macrophages produce large amounts of TNF following detection of bacterial-derived products such as Lipopolysaccharide (LPS), TNF rapidly drives innate immune reactions in response to bacterial infections. Indeed, septic shock as a result of chronic bacterial exposure is believed to be instigated by TNF (van der Poll and Lowry, 1995).

1.3.3 TNF as a driver of pathology

Multiple human pathophysiological conditions result from deregulated inflammatory responses. As TNF is a major driver of inflammation, it comes as no surprise that excessive TNF production is a contributory factor to many such conditions. Sepsis is a systemic state of chronic inflammation due to excessive stimulation of TLR-2 and TLR4, occurring as a result of severe infection (Angus and van der Poll, 2013; Liu and Malik, 2006). In 1985, it was shown that mice immunized with anti-TNF antibodies were protected from the lethal effect of bacterial lipopolysaccharide (LPS), identifying TNF as a major driver of septic shock (Beutler et al., 2008). This lead to the introduction of anti-TNF therapies, which dramatically improved the outlook for patients with diverse inflammatory diseases. Indeed, auto-immune diseases including rheumatoid arthritis, Chrohn's disease, inflammatory bowel disease (IBD) and psoriasis are driven, at least in part, by excessive TNF (Bradley, 2008).

Rheumatoid arthritis is characterised by inflammation of the synovium in joints, leading to cartilage destruction (Feldmann and Maini, 2001). Arthritis is worsened

by the accumulation of immune cells in the synovium that migrate towards elevated levels of cytokines and chemokines including IL-8, CXCL1, RANTES, and MCP-1, all of which are TNF-inducible (Brennan and McInnes, 2008). Indeed, administration of anti-TNF monoclonal antibodies to cultured cells from affected joints, attenuates spontaneous release of multiple chemokines and cytokines (Feldmann and Maini, 2001). Anti-TNF therapy is now widely used as an effective arthritis therapeutic with Infliximab (monoclonal antibody), and Etanercept (TNF-R-Fc fusion protein) representing billion dollar industries worldwide in this context, as well as other TNF-neutralizing agents.

Psoriasis is a common chronic inflammatory disease of the skin, characterised by abnormal epidermal growth, differentiation and immune cell infiltration, particularly Th-17 polarised T-cells (Maeda et al., 2012). Indeed, individuals with psoriatic lesions have increased TNF levels in the affected area and whole blood (Zaba et al., 2007). During psoriasis, TNF is known to stimulate production of other cytokines and interleukins, particularly IL-17, from keratinoctyes and Th-17 cells, creating a positive feed-back loop and worsening inflammation. Administration of TNF inhibitors has been demonstrated to alleviate symptoms by attenuating Th-17 T-cell involvement, thus breaking the inflammatory loop (Zaba et al., 2007).

While anti-TNF therapies have proven extremely effective in ameliorating inflammatory disease symptoms, particular patients depleted of TNF become vulnerable to opportunistic infection, particularly Tuberculosis (TB) (Keane et al., 2001). In contrast, overproduction of TNF during TB infection is detrimental, as it leads to macrophage necroptosis and bacterial escape into the extracellular space, facilitating spread of the infection (Roca and Ramakrishnan, 2013). These observations highlight the importance of TNF for effective bacterial control and the pathological consequences of its overproduction or deregulation.

1.3.4 The Ubiquitin system regulates inflammation and cell death

Ubiquitination of receptor complexes is essential for optimal activation of multiple downstream signaling cascades, as will be described for the TNF receptor complex below. Because activation of pathways such as NFκB promote inflammation and restrain cell death, ubiquitination is a core regulator of innate immunity and cell death processes.

Ubiquitination is a post-translational modification in which lysine residues on proteins are conjugated with ubiquitin chains. Ubiquitin is attached to the amino group of target protein lysine residues via its C-terminal glycine residue. This process occurs in a three-step enzymatic reaction. Firstly, an ubiquitin-activating enzyme (E1) attaches the ubiquitin molecule to the catalytic cysteine residue of an ubiquitin conjugating enzyme (E2). Ubiquitin ligases (E3), including RING ligases such as IAPs, then act as adaptors between the E2 and the target substrate, facilitating isopeptide bond formation between the ubiquitin molecule and the target substrate.

Single ubiquitin molecules (mono-ubiquitin) or linkages of multiple ubiquitin molecules (poly-ubiquitin) can be conjugated to target proteins. While multiple varieties of ubiquitin linkages have been identified, three major forms of ubiquitin linkages are involved in inflammatory signaling cascades and cell death; lysine-63 (K63) linked, lysine-47 (K47) linked and linear (M1) linked. K63 linked ubiquitin chains typically form signaling platforms that attract kinases and other molecules that initiate signal transduction pathways. K47 linked ubiquitin acts as a signal to initiate proteasomal degradation of the target protein. This process is important for protein turnover but can also activate inflammatory signaling pathways as described for IKBα above. M1 linked ubiquitin chains are assembled by a dedicated E3 ligase complex called LUBAC, as mentioned above. Linear ubiquitin linkages are thought to serve as signaling platforms, similarly to K63 linked chains. Indeed, ubiquitination is a reversible process. Ubiquitin linkages can be disassembled by a specific family of proteins called deubiquitinases (DUBs). As Ubiquitin chains serve as pathway

activation platforms, DUBs provide a method of fine-tuning or even switching off such ubiquitin-driven signaling cascades.

1.3.5 Assembly of the TNFR 'pro survival' signaling complex 'I'

TNF induces rapid formation of a pro-survival, pro-inflammatory 'complex I' (Micheau and Tschopp, 2003). Initially, TRADD is recruited to the intra-cellular, death domain (DD) containing cytoplasmic tails of trimerised receptors via homotypic DD-DD interactions. TRADD facilitates binding of RIPK1, which also occurs via the DD present in RIPK1. TRAF2 is also recruited to specific binding sites on TRADD. TRAF2 facilitates recruitment of cIAP1 and cIAP2 via a cIAP1/2-interacting motif, completing the make-up of the core TNFR signaling platform (Vince et al., 2009) (Figure 1.4). The E3 ubiquitin ligases TRAF2 and cIAPs then conjugate K63 linked poly-ubiquitin chains on RIPK1 (Alvarez et al., 2010; Bertrand et al., 2008; Mahoney et al., 2008; Varfolomeev et al., 2008), in association with the ubiquitin conjugating enzyme (E2), UbcH5a (Varfolomeev et al., 2008). The involvement of the cIAPs at this stage of complex formation is of extreme importance as it promotes inflammatory signaling and prevents RIPK1 from driving apoptosis (Feoktistova et al., 2011; Tenev et al., 2011; Wang et al., 2008).

RIPK1 poly-ubiquitin chains provide a scaffolding platform for TAK1 in association with TAB1 and TAB2 (TAK/TAB complex) and the IKK complex containing kinases IKK1, IKK2 and a regulatory subunit NEMO (IKKγ). A complex of HOIL1, HOIP and Sharpin termed the LUBAC complex also ubiquitinate RIPK1 and NEMO through linear (M1) linkages, among other receptor components, to stabilize the pro-inflammatory signaling complex (Gerlach et al., 2011; Haas et al., 2009; Tokunaga et al., 2009). The TAK1/TAB complex in combination with MEKK3 then activates the IKK complex by phosphorylation (Blonska et al., 2005; Wang et al., 2001). Phosphorylation of IKBα by IKK2 results in rapid conjugation of K48 ubiquitin linkages on IKBα, targeting it for proteasomal degradation. Under steady state conditions, IKBα sequesters canonical NFκB subunits (p50/p65) in the cytoplasm, which proceed to the nucleus following TNF stimulation and subsequent IKBα degradation, to drive inflammatory gene transcription. RIPK1-associated ubiquitin chains can be removed by the deubiquitinases (DUBs) cylindromatosis (CYLD) and

the A20 family member Cezanne (Enesa et al., 2008; Kovalenko et al., 2003; Wilson et al., 2009). As these enzymes are NFκB targets, they operate in a negative feedback loop to provide an additional level of control on signal intensity.

NFκB drives the transcription of multiple TNF-inducible genes (Zhou et al., 2003). TNF 'complex 1', however, also initiates activation of multiple MAPK cascades including p38, MEK/ERK and JNK. MAPKs are a group of enzymes that control a variety of cellular processes in response to diverse extracellular stimuli (Karin, 1998) and are known to participate in TNF-induced chemokine production, proliferation and differentiation (Hirano et al., 2003; Radeff-Huang et al., 2007; Yanagawa et al., 2002). TNF-induced p38, ERK and JNK require RIPK1 and TRAF2 for optimal activation (Devin et al., 2003). In fact, unlike the other MAPKs, ERK activation requires RIPK1 kinase activity (Devin et al., 2003). The isoforms of p38 are predominantly activated by MKK3 and MKK6, while p38α can also auto-activate when associated with TAB1 (Ge et al., 2002; Zarubin and Han, 2005). ERK appears to be activated by RIPK1 kinase activity towards MKK1 and MKK2 (Devin et al., 2003; Robinson and Cobb, 1997), while JNK activation is mediated by MKK4 and MKK7 (Moriguchi et al., 1997; Tournier et al., 2001) (Figure 1.4).

1.3.6 TNF-Induced apoptosis

The extrinsic apoptotic pathway is activated by the binding of extracellular ligands, often FasL, or TRAIL to trans-membrane 'death receptors'. Death domain-containing adapter protein FADD is then recruited to the cytoplasmic tails of trimerized receptors which in turn results in the recruitment of pro-caspase-8 to form the death-inducing signaling complex. Pro-caspase-8 is activated by auto-processing and initiates cellular demise through proteolytic cleavage and activation of executioner caspases-3 and -7 (Ashkenazi and Dixit, 1998). While the TNF receptor is regarded as a member of the death receptor family, TNF stimulation alone does not usually result in apoptosis. If NFκB activation is blocked, however, or the cIAPs are removed, TNF can induce formation of 'Complex II' composed of RIPK1, FADD and caspase-8, resulting in apoptosis via a similar mechanism to Fas or TRAIL (Micheau and Tschopp, 2003) (Figure 1.5). Upon NFκB inhibition, levels of the endogenous caspase-8 inhibitor cFLIP are lowered, thus reducing the

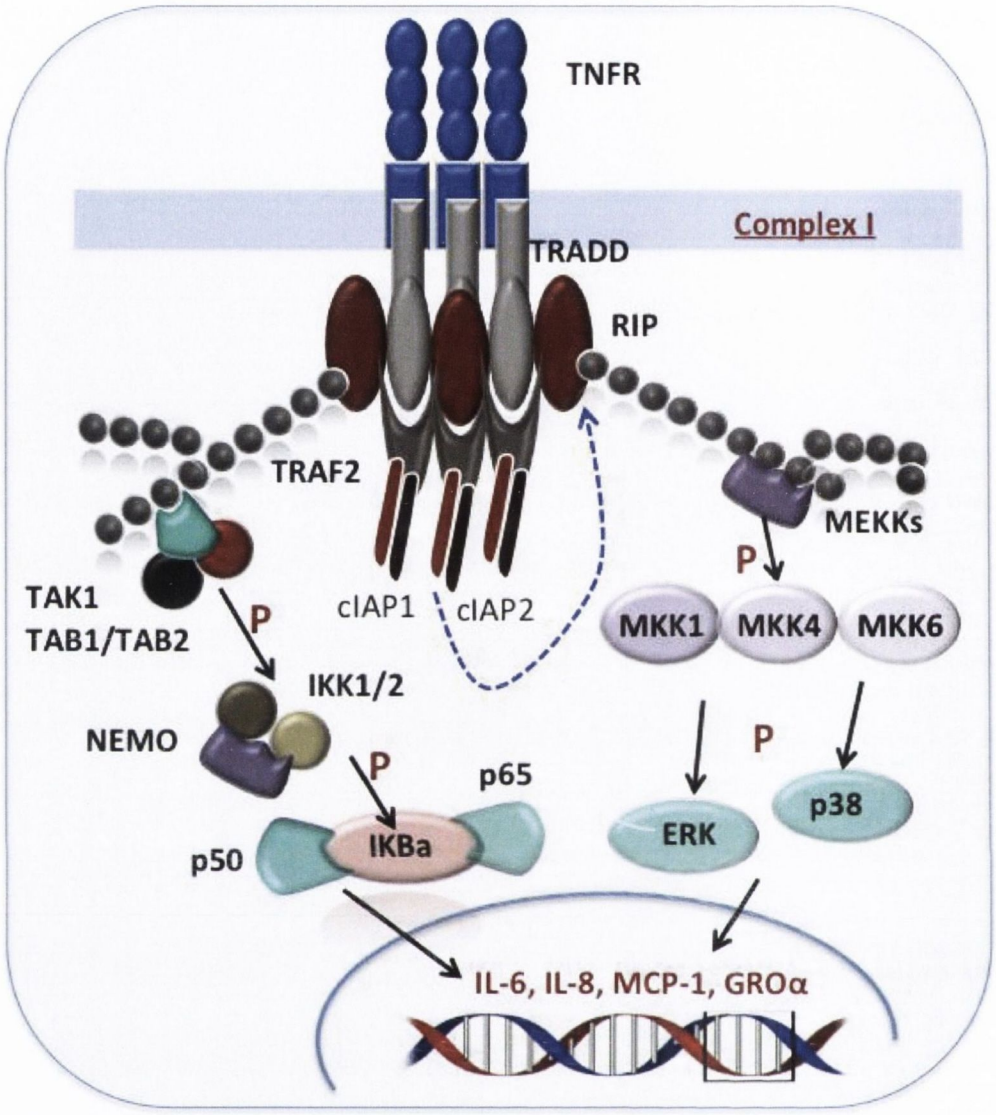

Figure 1.4

Assembly of the pro-survival, pro-inflammatory TNFR 'complex I'

TNF stimulation results in TRADD recruitment to trimerised tails of the TNF receptor. RIPK1 is recruited to TRADD via death domain interactions. RIPK1 then facilitates binding of TRAF2, which in turn recruits the cIAPs. cIAPS and possibly TRAF2 ubiquitinate RIPK1 with K63 linkages, providing a scaffold for binding of TAK1 and other MKKs. TAK1 then activates the IKK complex, which phosphorylates IKBα, triggering its proteasomal degradation. This releases NFkB P50 and p65 subunits to drive inflammatory gene transcription and pro-survival molecules such as cFLIP and cIAPs. Meanwhile, MKK1, MKK4 and MKK6 activate ERK and p38 MAPKs, which also drive cytokine production and alternative processes such as proliferation and differentiation.

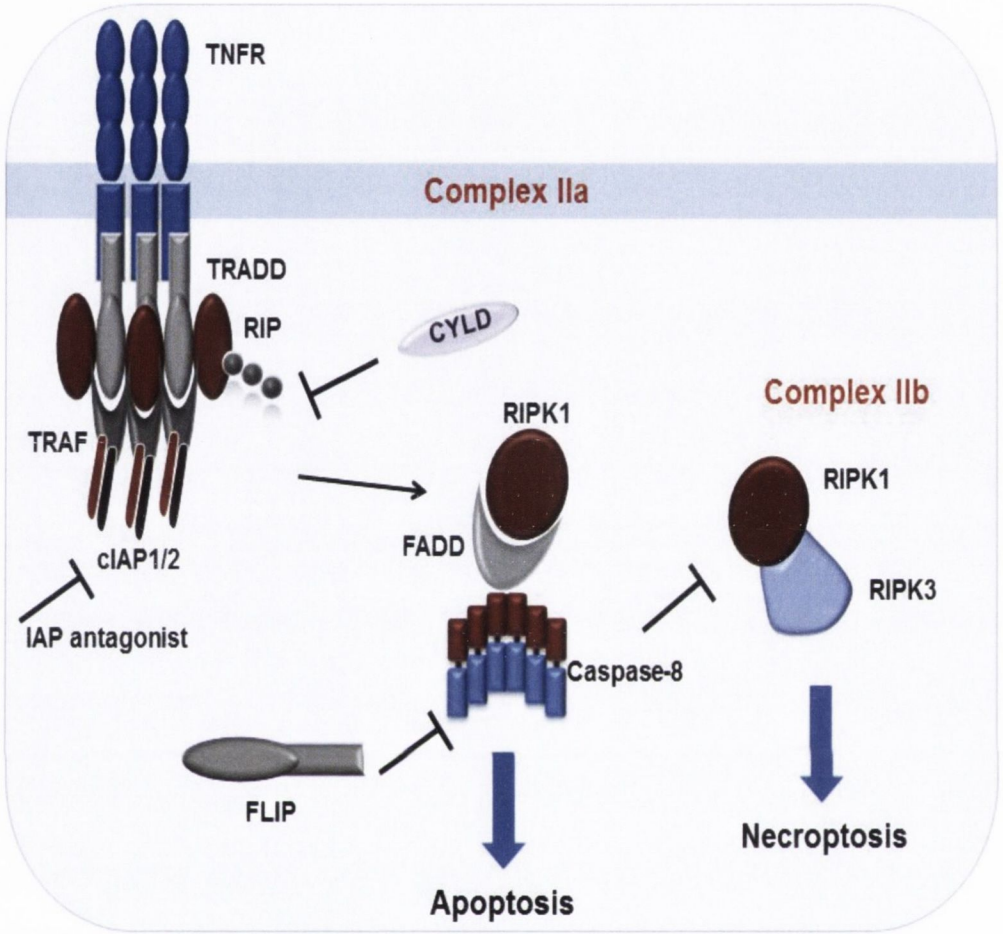

Figure 1.5

Assembly of the TNF-induced Death Inducing Signaling Complex (DISC), 'complex II'

TNF stimulation under normal circumstances results in NFkB activation in the absence of cell death via complex I. However, if IAPs are removed, RIPK1 fails to be ubiquitinated and the apoptosis-inducing DISC is formed, composed of RIPK1 FADD, Caspase-8 and cFLIP in the cytosol. Furthermore, excessive activity of the RIPK1 deubiquitinase CYLD can promote DISC assembly. cFLIP restrains activation of caspase-8 to levels that would induce apoptosis. Caspase-8 however, still retains enough proteolytic activity to cleave and inactivate RIPK1 and RIPK3 in complex IIb, also referred to as the necrosome. If Caspase-8 is inhibited or deleted, complex 11b can be formed from complex IIa, leading to RIPK3 activation and necroptosis.

threshold for TNF-induced apoptosis. As IAPs are also required for optimal TNF-induced NFκB activation (Mahoney et al., 2008; Varfolomeev et al., 2007), removing them with an IAP antagonist also results in a lower apoptotic threshold via cFLIP down regulation. Furthermore, if IAPs are depleted, RIPK1 fails to be ubiquitinated also becomes liberated from the activated TNF receptor complex I. Under these circumstances, RIPK1 forms a caspase-8 activating complex comprised of RIPK1, FADD and Caspase-8 to drive apoptosis (Wang et al., 2008) (Figure 1.5).

1.3.7 Lessons from TNF or TNFR knockout mice

The importance of TNF in immunity is evident from analyzing the immune-competence of TNF knockout mice. While mice lacking either *TNF* or *TNF-R1* develop normally, they have a severely impaired innate immune system but maintain a relatively intact adaptive immune system (Marino et al., 1997). T-cell proliferation, cytotoxicity and cytokine secretion is normal, however, $TNF^{-/-}$ mice fail to develop organized B-cell germinal centers (Marino et al., 1997; Pasparakis et al., 1996). B-cell-derived immunoglobulin (IgG) to thymus-independent antigens is normal but slightly impaired to thymus-dependent antigen. Furthermore, macrophage phagocytic activity is relatively normal (Marino et al., 1997). Importantly, however, $TNF^{-/-}$ or $TNFR^{-/-}$ mice are highly susceptible to infection with pathogens including *Candida albicans*, *Corynebacterium parvum*, *Mycobacterium tuberculosis* and *Lysteria monocytogenes* (Flynn et al., 1995; Marino et al., 1997; Rothe et al., 1994). This highlights the importance of TNF in driving the innate immune response to control initial pathogen challenge.

1.4 RECEPTOR INTERACTING PROTEIN KINASES (RIPKS)

1.4.1 Introduction to kinase activity

Kinases are a large and diverse family of enzymes comprised of 518 members in the human genome, collectively referred to as the kinome (Manning et al., 2002). Kinases transfer phosphate groups from high energy molecules, such as ATP, to specific residues on target proteins in a process called phosphorylation. Kinases are critically involved in multiple cellular processes, particularly signal transduction

events. Kinase-mediated phosphorylation of target proteins can manipulate target function by altering activity, stability, localization or by changing binding propensity to other molecules. Protein kinases can be grouped according to the target residue they phosphorylate. These include serine, threonine, tyrosine and histidine kinases (Manning et al., 2002).

Kinase target specificity must be a highly regulated process to maintain cellular homeostasis and prevent oncogenic transformation. Indeed, a single kinase must distinguish between over 700,000 potential substrates (Ubersax and Ferrell, 2007). The first level of specificity arises from the kinase enzymatic active site. Shape, charge and hydrophobicity of the active site can increase the propensity of a kinase to bind a substrate with complementary characteristics. Secondly, kinases often contain docking sites, adjacent to the enzymatic active site. These conserved regions guide kinases to their preferred target substrates. Thirdly, cellular localization of kinases can increase their concentration in a particular cell compartment, increasing their availability to phosphorylate substrates. Indeed, kinase activation can lead to their re-localization in this context. Finally, scaffolding proteins can provide an extra level of control on kinase specificity. Specific adaptor proteins can form complexes with kinases and their substrates, facilitating kinase-substrate interactions (Ubersax and Ferrell, 2007). Indeed, the specificity of kinases enables the design of small molecules to inhibit particular kinases in biological processes.

Kinases are often activated in a cascade effect, with one upstream kinase phosphorylating and activating another (Adams, 2001). These cascades often culminate in phosphorylation of transcription factors that drive gene induction, such as NFkB subunits. However, kinases can also auto-activate by self-phosphorylation. This often occurs if same kinases are aggregated in close proximity (Adams, 2001). As receptors, such as TNFR, are activated through trimerization, recruited kinases can be brought into close proximity by adaptor molecules. Similarly, receptor-associated ubiquitin chains can promote kinase clustering and activation (Adhikari et al., 2007).

1.4.2 Receptor interacting protein kinase 1 (RIPK1)

RIPK1 is a serine/threonine protein kinase belonging to the RIP kinase family. RIPK1 can be considered the central player in promoting TNF-driven NFκB activation and inflammation as described above. Indeed, cells derived from RIPK1 null animals fail to efficiently activate NFκB in response to TNF (Kelliher et al., 1998). RIPK1 was initially described in 1995 as a Fas receptor binding protein via its C-terminal death domain (Stanger et al., 1995) (Figure 1.6). As with all RIP kinase family members, RIPK1 has an active kinase domain at its N-terminus. The kinase activity of RIPK1 is thought to be required for TNF-induced necroptosis, by phosphorylating RIPK3 (He et al., 2009), but dispensable for TNF-induced NFκB activation (Newton et al., 2004). RIPK1 also contains a RHIM domain, which facilitates interaction with RIPK3, the consequences of which will be discussed below. RIPK1 is now known to associate with many TNF super family receptors, toll-like receptors 3 and 4 and cytoplasmic PRRs such as RIG-I/MDA5. Indeed, RIPK1 promotes TRIF- or IPS-1-dependent inflammatory gene induction following engagement of TLR3, TLR4 or RIG-I pathways (Festjens et al., 2007; Kawai and Akira, 2005). Similarly, RIPK1 null Jurkat cells are compromised in NFκB activation in response to Fas ligand (Kreuz et al., 2004). Interestingly, RIPK1 also controls cytokine production in response to DNA damage and mediates formation of the cell death inducing 'Ripoptosome' platform upon cIAP loss or DNA damage (Biton and Ashkenazi, 2011; Feoktistova et al., 2011; Ofengeim and Yuan, 2013; Tenev et al., 2011). Taken together, RIPK1 is a central participant in driving inflammation or cell death, in response to diverse stimuli.

Because RIPK1 activity drives cell death or inflammation depending on the circumstances, it requires tight post-translational regulation. During TNF-induced apoptosis, RIPK1 is cleaved by caspase-8 which diminishes its NFκB promoting activity and produces a product that improves association with FADD (Lin et al., 1999; Martinon et al., 2000). The failure of cleaved RIPK1 to activate NFκB then initiates an amplification loop to drive apoptosis. This process is now known to prevent RHIM domain-dependent association with RIPK3, ensuring that death proceeds by caspase-dependent apoptosis and not RIPK3-dependent necroptosis

(He et al., 2009; Ofengeim and Yuan, 2013). Furthermore, Caspase-8 constitutively cleaves RIPK1 to prevent activation of the viral sensing RIGI/MDA5 pathway (Rajput et al., 2011). Similarly, caspase-8 cleaves RIPK1 during macrophage differentiation in response to M-CSF (Rebe et al., 2007). Here, caspase-8 was shown to associate with RIPK1, FADD and FLIP leading to NFκB activation and cell differentiation. Caspase-8 mediated cleavage of RIPK1 attenuates NFκB activation, preventing excessive inflammation after completion of the differentiation process (Rebe et al., 2007). RIPK1 is also cleaved by bacterial proteases during infection (Madrigal et al., 2012). This is likely a pathogen strategy to terminate RIPK1-driven inflammation following bacterial detection. RIPK1 activity, therefore, is tightly regulated, particularly by caspase-8, to prevent deregulation of multiple signaling pathways.

1.4.3 Receptor interacting protein kinase 3 (RIPK3)

RIPK3 is a Serine/Threonine protein kinase with similar structure to RIPK1 (Figure 1.6). It does, however, lack the N-terminal death domain present in RIPK1, preventing it from directly associating with other death domain containing molecules, such as TRADD or FADD. RIPK3 was initially identified as a protein capable of driving apoptosis and NFκB when overexpressed (Pazdernik et al., 1999; Sun et al., 1999; Yu et al., 1999). While *RIPK1*$^{-/-}$ cells fail to activate NFκB in response to TNF and deletion results in immune compromised, perinatal lethal mice (Kelliher et al., 1998; Ting et al., 1996), *RIPK3*$^{-/-}$ cells display normal NFκB activation and mice remain viable with no gross phenotype (Newton et al., 2004). These results suggest a possible model whereby RIPK3-driven NFκB activation is tightly regulated and occurs only upon breach of an activation threshold, or upon deregulation or genetic loss of proteins that usually restrain RIPK3 activity. Indeed, myeloid lineage-specific deletion of XIAP and cIAPs, results in spontaneous TNF-secretion that is attenuated by combined loss of RIPK3. This suggests IAPs restrain RIPK3 from participating in cytokine production (Wong et al., 2014). Similarly to RIPK1, RIPK3 is processed by caspase-8, rendering it a pro-apoptotic molecule and preventing necroptosis (Feng et al., 2007; Zhang et al., 2009). Thus, if caspase-8 activity is inhibited, RIPK3 can become dereregulated, resulting in necroptosis as will be discussed.

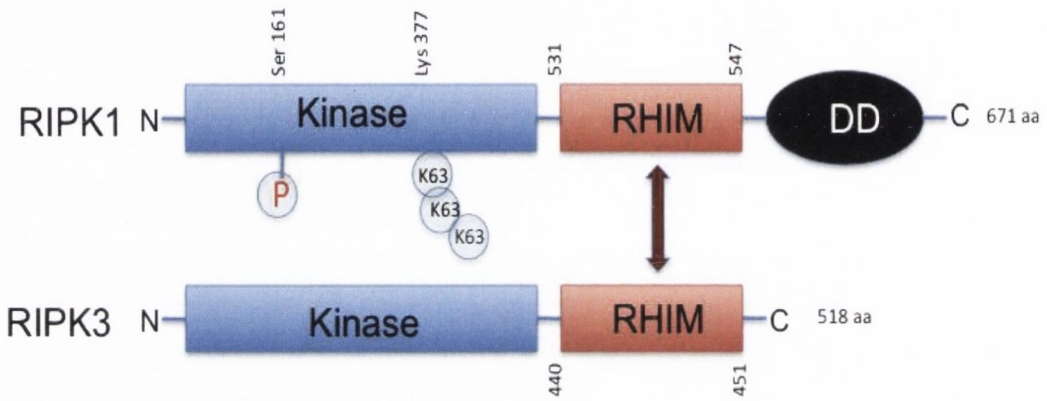

Figure 1.6

Domain structure of the related kinases RIPK1 and RIPK3

RIPK1 and RIPK3 are structurally similar Serine/Threonine protein kinases. Each possesses a kinase domain at the N-terminal while only RIPK1 has a C-term death domain, facilitating binding to TRADD or FADD. During inflammatory TNF signaling, cIAPs conjugate K63 linked ubiquitin chains on to RIPK1 at Lysine 377, to promote NFkB activation.During programmed necrosis, RIPK1 is phosphorylated at Serine 161 while RIPK3 is phosphorylated by RIPK1 at Serine 227. RIPK1 and RIPK3 interact through a phosphorylation driven association of the RHIM domains.

1.5 THE INHIBITOR OF APOPTOSIS PROTEINS (IAPS)

1.5.1 Overview of IAPs

The inhibitor of apoptosis proteins (IAPs), are a related family of proteins, comprised of eight members, that have diverse cellular functions. Functions of IAPs include regulation of apoptosis, mitosis, migration and inflammation (Gyrd-Hansen and Meier, 2010; O'Riordan et al., 2008; Silke and Brink, 2010). IAPs were initially identified as virus-encoded caspase inhibitors (Birnbaum et al., 1994; Crook et al., 1993), produced to prevent host cell death and prolong viral replication time. Mammalian IAPs, therefore, were long considered to be biological inhibitors of caspase activity, providing an extra level of control to the apoptotic program. XIAP is now known to bind and inhibit caspase-9, caspase-3 and caspase-7 activity (Gyrd-Hansen and Meier, 2010). While cIAP1 and cIAP2 are capable of binding caspases through similar domain structure to XIAP, they are poor inhibitors of caspase activity (Eckelman and Salvesen, 2006). It is becoming increasingly apparent, however, that the IAPs implicated in cell death control, cIAP-1, cIAP-2 and XIAP, are also critical mediators of signal transduction events that promote inflammation (Bertrand et al., 2008; Damgaard et al., 2012; Vince et al., 2012).

IAPs are defined by the presence of one or more baculoviral IAP repeat (BIR) domains, which can interact with proteins that harbor an IAP Binding Motif (IBM), such as caspases and smac/diablo (Figure 1.7). For example, the BIR2 of XIAP interacts with the IBM of caspase-3 and-7, inhibiting their activation (Scott et al., 2005). cIAP1/2 and XIAP also possess a RING domain with E3 ubiquitin ligase activity, which promotes attachment of poly-ubiquitin chains on RIPK1 or RIPK2 respectively, to promote inflammatory signaling pathways (Figure 1.7). They also contain a ubiquitin-association (UBA) domain, which binds mono, K63 and K48 linked ubiquitin chains and probably aids association with the proteasome for degradation (Blankenship et al., 2009) and with ubiquitin-decorated signaling complexes (Gyrd-Hansen et al., 2008).

1.6.2 IAP antagonists (smac mimetics)

The mitochondrial protein SMAC/DIABLO is released from within the mitochondrial membrane space during apoptosis, which binds and degrades XIAP, cIAP1 and cIAP2. This process is thought to aid in caspase activation and subsequent

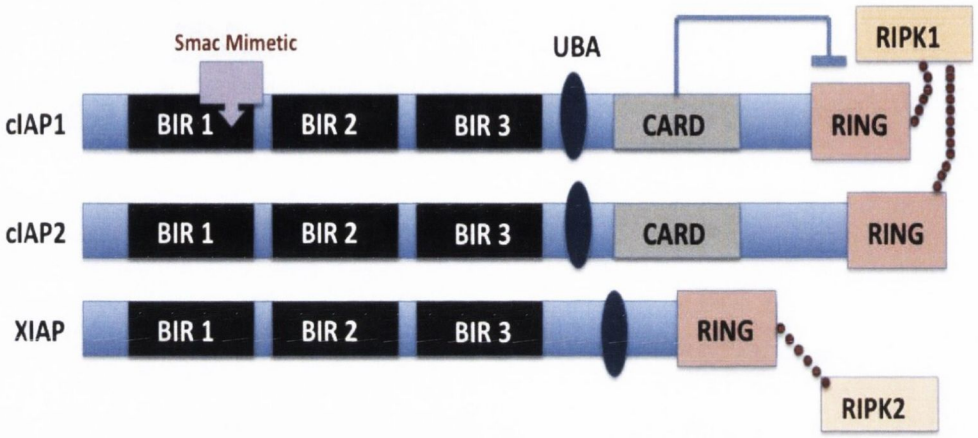

Figure 1.7

Domain structure of cIAP1, cIAP2 and XIAP

IAPs are defined by the presence of at least one Baculovirus IAP Repeat (BIR) domains. cIAP1, cIAP2 and XIAP have three BIR domains. Each of these IAPs also has a Ubiquitin Association Domain (UBA), which facilitates IAP binding to a variety of ubiquitin linkages. The RING domain is an provided IAPs with E3 ubiquitin ligase activity. During TNF signaling, the RING of cIAP1 and cIAP2 conjugate K63 linked ubiquitin on to RIPK1, while XIAP acts similarly towards RIPK2 following NOD receptor stimulation. cIAP1 and cIAP2 have a Caspase Activation and Recruitment Domain (CARD). The CARD restrains autoactivation of the RING domain. Small molecule IAP antagonists (smac mimetics) bind to IAP BIR domains. This triggers RING dimerization, activation and self ubiquitination with K47 linkages, promoting proteasomal degradation.

apoptosis (Shi, 2002; Wu et al., 2000). Given that IAPs are often up-regulated in cancer and are thought to facilitate chemotherapeutic resistance, small molecule mimetics of SMAC/DIABLO (smac mimetics), were developed to antagonize IAPs (LaCasse et al., 1998). Indeed, IAP antagonists have been shown to rapidly trigger IAP degradation, increasing tumor cell sensitivity to chemotherapeutic drugs by neutralizing XIAP-mediated apoptosis resistance (Eschenburg et al., 2012). Importantly however, IAP antagonists can also render tumor cells susceptible to TNF-induced apoptosis by eliminating cIAP1 and cIAP2 (Varfolomeev et al., 2007; Vince et al., 2007). Mechanistically, smac mimetic binding to the BIR domains of IAPs triggers auto-ubiquitination by the C-terminal RING domain, targeting the IAP for proteasomal degradation (Feltham et al., 2011; Varfolomeev et al., 2007) (Figure 1.7). While IAP antagonists were developed to sensitize cancer cells to apoptosis, they have been extremely useful in probing the involvement of IAPs alternative cellular processes. Indeed, IAPs are now known to be required for optimal TNF- and PRR-induced NFκB activation as a result of the availability of smac mimetics (Damgaard and Gyrd-Hansen, 2011; Mahoney et al., 2008).

1.6.3 IAPs dictate the switch between TNF-induced inflammation and apoptosis

cIAP1 and cIAP2 often dictate the outcome of TNF receptor stimulation. As mentioned above, TNF alone does not usually kill the vast majority of cell types. Indeed, cIAPs promote inflammatory signaling in the absence of cell death by ubiquitinating RIPK1. However, If IAPs are removed genetically or with an IAP antagonist, TNF stimulation can be converted to an apoptotic signal (Takahashi et al., 2014; Wang et al., 2008). Upon IAP removal in some cell types, RIPK1 fails to initiate death protective NFκB signaling and forms a complex with FADD and caspase-8 (Bertrand et al., 2008; Varfolomeev et al., 2008; Wang et al., 2008). Proximity-induced auto activation of caspase-8 then results in apoptotic cell death (figure 1.5). Knockdown of RIPK1 in the absence of IAPs protects from TNF-induced apoptosis, highlighting the importance of RIPK1 in this context (Petersen et al., 2007; Wang et al., 2008). Interestingly, RIPK1 is not required for TNF-dependent apoptosis following NFκB inhibition and a subsequent reduction in cFLIP expression (Wang et al., 2008), demonstrating the complex relationship between

cIAPs and RIPK1 in determining cell death.

The cIAPs have also been shown to prevent spontaneous formation of a large, cytoplasmic, cell death inducing platform containing RIPK1, FADD and caspase-8, called the 'Ripoptosome' (Feoktistova et al., 2011; Tenev et al., 2011). Indeed, IAP depletion is sufficient for Ripoptosome formation as it occurs in the absence of TNF, Fas or TRAIL stimulation. Upon Ripoptosome formation, however, stimulation with TNF family ligands or toll ligands triggers Ripoptosome association with the receptor complex, leading to caspase-8 activation and apoptosis.

It is important to note however, that sensitization to apoptosis as a result of IAP depletion, is primarily observed in transformed cells. Many primary cells are TNF responsive (they secrete chemokines upon TNF stimulation) but are not sensitized to TNF-induced apoptosis upon IAP depletion, for reasons that are not fully clear. However, as IAPs ubiquitinate RIPK1 to provide an NFκB activation platform, loss of IAPs can attenuate inflammatory signaling pathways in response to TNF, FAS and toll ligands (Cullen et al., 2013; Damgaard and Gyrd-Hansen, 2011; Mahoney et al., 2008). Thus, IAPs are critical in driving TNF-induced inflammation, while repressing TNF-induced apoptosis.

1.6.4 IAPs prevent non-canonical NFκB activation

The non-canonical NFκB pathway is activated by the kinase NIK. NIK is constitutively turned over by recruitment to a TRAF2, TRAF3 and cIAP1/2-containing complex . Within this complex, cIAPs conjugate K47 linked ubiquitin on to NIK, targeting it for proteasomal degradation (Sun, 2012). If cIAPs are removed, NIK levels accumulate, allowing it to phosphorylate IKK1. IKK1 phosphorylates p100, which is then processed to release active NFκB subunits P52/RelB (Darding and Meier, 2012) (Figure 1.8). Some cancer cell lines undergo apoptosis in response to a smac mimetic alone. In these cells, IAP degradation was shown to initiate NIK- dependant non-canonical NFκB activation which produced TNF, acting in an autocrine loop to render cells sensitive to the TNF/smac mimetic induced apoptosis (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). While this phenomenon may be very useful therapeutically for cancer, the

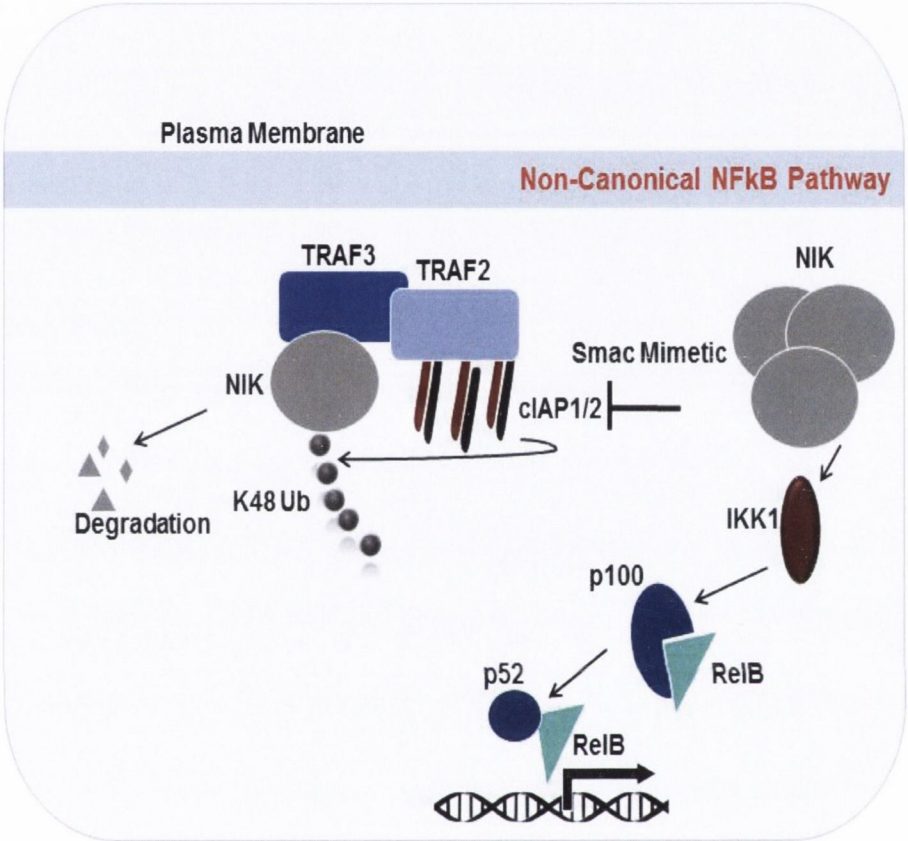

Figure 1.8

cIAPs repress activation of the non-canonical NFκB pathway

Under normal conditions, NIK is bound by TRAF3 and recruited to cIAP1/2 via binding to TRAF2. This TRAF2/3 cIAP1/2 complex mediates constant ubiquitination and proteasomal degradation of NIK. If cIAPs are removed, NIK levels accumulate, leading to phosphorylation and activation of IKK1. IKK1 phosphorylates p100, stimulating its processing to p52, and release of RelB. P52 and RelB transcription factors then migrate to the nucleus to drive non canonical NFkB genes. RelB and p52 can act as homodimers or heterodimers.

inflammatory consequences of TNF secretion from tumor cells and surrounding primary cells following IAP loss must be considered. Furthermore, it remains to be examined if smac mimetic-induced non-canonical NFκB activation drives cytokines other than TNF and promotes inflammation in this context.

1.6.5 Lessons from IAP knockout mice

Double knockout of cIAP1 and cIAP2 results in embryonic lethality at E10.5 due to cardiac abnormalities (Moulin et al., 2012). Importantly, combined deletion of the TNF receptor rescues these mice, highlighting the importance of the TNF/RIPK1/cIAP axis in cell death regulation. MEFs from these mice only required cIAP1 for efficient NFκB activation and protection from death, suggesting a redundant role in protecting from cell death. While cIAP1 single knock out mice may appear normal, however, they fail to clear lung infection by *C. pneumoniae* (Prakash et al., 2009), demonstrating a non-redundant role during infection. In this context, macrophage recruitment to the infected lung was delayed in the absence of cIAP1, potentially due to a deficiency in chemotactic cytokine release. As mentioned above, cIAPs serve to protect from cell death by promoting RIPK1 ubiquitination. In line with this model, mice lacking either cIAP1 or cIAP2 display increased macrophage necroptosis upon infection with *Listeria Monocytogenes*, demonstrating that cIAPs restrain not only apoptosis, but also necroptosis (McComb et al., 2012).

1.6 NECROPTOSIS

1.6.1 Introduction to necroptosis

Necrosis has long been thought to represent a deregulated form of cell death, usually occurring as a result of physical insult and resulting in membrane rupture (Kono and Rock, 2008; Matzinger, 1994). Recent developments, however, have led to the realisation that necrosis can proceed under the control of specific signalling pathways. It had been known for some time that certain cell types died in a necrotic like fashion in the presence of caspase inhibitors (Lemaire et al., 1998), suggesting apoptosis is the preferred death mode and necrosis is a 'failsafe' mechanism if caspase activation is frustrated. Later, RIPK1 was shown to be required for this outcome in Jurkat T-cells treated with Fas and zVAD (Holler et al., 2000). Following

the discovery of a small molecule inhibitor of this caspase-independent cell death, Necrostatin-1, the cell death mode was termed necroptosis (Degterev et al., 2005). Indeed, RIPK1 was later identified as the cellular target of Necrostatin-1, placing RIPK1 as a central participant in the necroptosis pathway (Degterev et al., 2008). As necrosis can trigger sterile inflammation, regulated necrosis is thought to also be a pro-inflammatory cell death mode *in vivo* (Kaczmarek et al., 2013), as will be discussed below.

1.6.2 Signaling to necroptotic cell death

Necroptosis is thought to result from TNF-, Fas-, TRAIL- or TLR-induced hyperactivation of the RIPK3 kinase. Generally, RIPK1 is thought to phosphorylate RIPK3 following receptor stimulation, leading to RIPK3 activation and oligomerization (He et al., 2009; Sun et al., 2012). Oligomerized RIPK3 then recruits and activates the pseudokinase MLKL (Sun et al., 2012). The pseudokinase domain of MLKL acts as a molecular latch, restraining activity of the N-terminal four-helix bundle (4HB), prior to phosphorylation by RIPK3 (Hildebrand et al., 2014). Upon this activation event, MLKL oligomerizes and relocates to plasma membranes in high molecular weight complexes (Cai et al., 2014; Dondelinger et al., 2014; Wang et al., 2014) (Figure 1.9). Membrane bound MLKL promotes necroptosis by a mechanism that is not fully understood, but may involve hyper-activation of the cation channel TRPM7, causing rapid ion influx and necrosis. Alternatively, oligomerized MLKL may directly drive membrane rupture through binding to phosphatidylinositol phosphates and cardiolipin via a cluster of positively charged amino acids in the MLKL N-terminal 4HB (Dondelinger et al., 2014; Wang et al., 2014). RIPK3 and MLKL appear to be essential for necroptosis, as cells lacking either fail to engage this process in response to all stimuli (He et al., 2009; Murphy et al., 2013; Sun et al., 2012).

1.6.3 Caspase-8 activity restrains necroptosis

Necroptosis usually occurs following TNF-like cytokine stimulation if caspase-8 activity is inhibited (Figure 1.9). This can be achieved experimentally by using the poly caspase inhibitor zVAD, but may occur *in vivo* due to expression of viral-

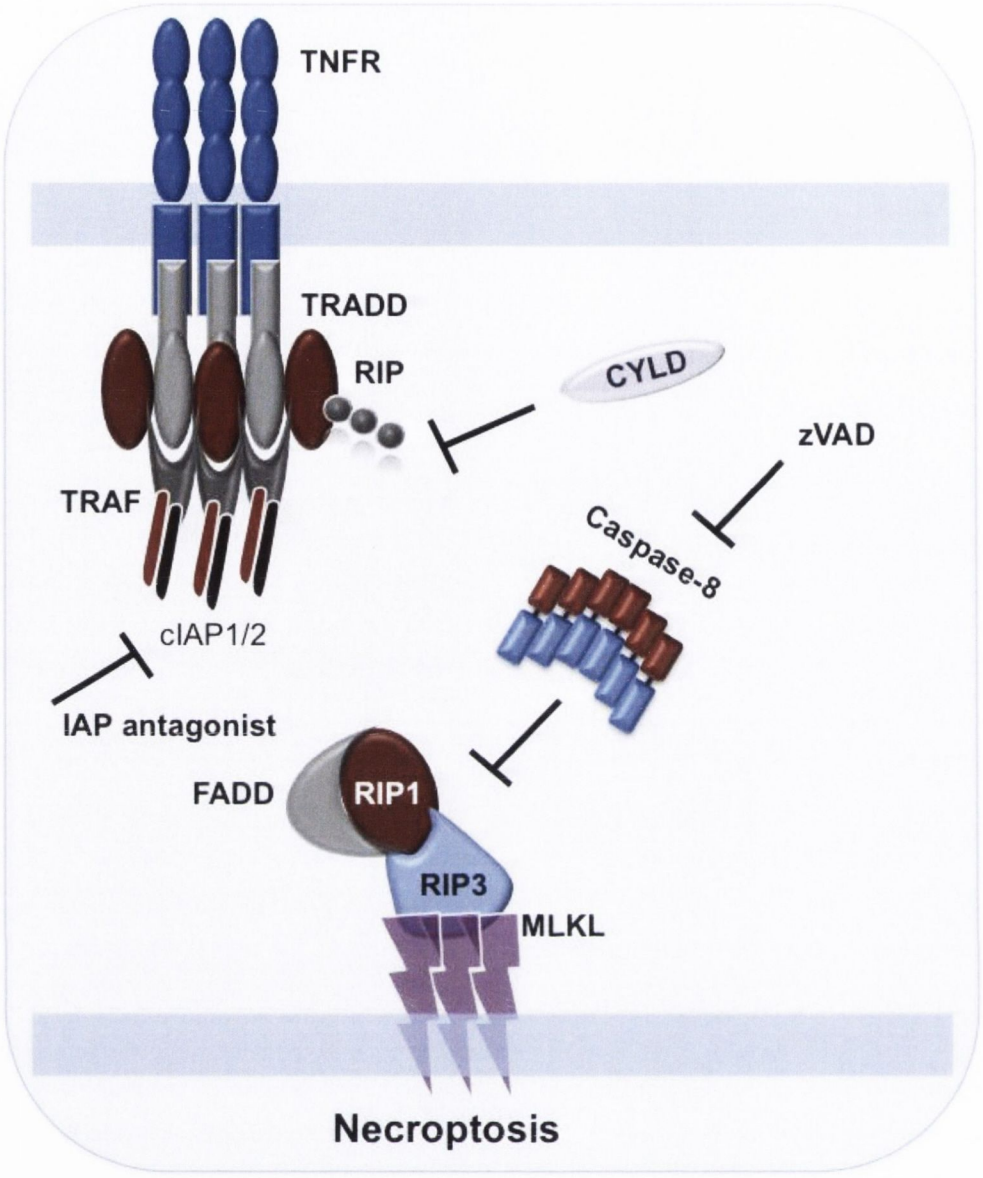

Figure 1.9

Caspase-8 prevents TNF stimulation from being converted into a necroptotic signal

Under normal circumstances, TNF stimulation promotes ubiquitination of RIPK1, which drives inflammatory cytokine transcription. However, if RIPK1 becomes de-ubiquitinated due to excessive CYLD activity, loss of IAPs or a block on NFkB activity, an apoptosis-inducing complex forms, comprised of FADD, RIPK1 and RIPK3. Caspase-8 activity prevents necroptosis in this setting by cleaving and inactivating RIPK1 and RIPK3, promoting apoptosis. However, if caspase-8 activity is blocked, RIPK1 and RIPK3 become deregulated and activate MLKL by phosphorylation. Activated MLKL migrates to the plasma membrane where it executes necrotic cell death, possibly be hyperactivating the cation channel TRPM7.

derived caspase inhibitors such as CrmA. Necroptosis is thought to occur if RIPK1 is insufficiently ubiquitinated, which would normally restrain RIPK3 association with a receptor complex. In the absence of sufficient caspase-8 activity, RIPK1 associates with RIPK3 and FADD in a cytosolic complex termed the necrosome (Vandenabeele et al., 2010), resulting in MLKL activation and necroptosis (Figure 1.9). Because IAPs ubiquitinate RIPK1, they prevent necrosome formation and are therefore potent inhibitors of necroptosis. Indeed, some cell types are resistant to necroptosis induced by TNF and zVAD alone, but can be sensitised by co-exposure to an IAP antagonist (He et al., 2009; Tenev et al., 2011).

Activation of the necroptosis pathway is tightly regulated. Following receptor stimulation, caspase-8 restrains activation of RIPK3 in two ways. Firstly, by cleaving the RIPK3-activating kinase RIPK1, and RIPK3 itself, as mentioned above (Ofengeim and Yuan, 2013). Secondly, caspase-8 cleaves the deubiquitinating enzyme CYLD (O'Donnell et al., 2011). CYLD negatively regulates cIAP mediated RIPK1 ubiquitination, thus antagonising RIPK1- mediated NFκB activation and cell survival. Consequently, deregulated CYLD activity can lead to excessive deubiquitination of RIPK1 and excessive recruitment and activation of RIPK3 (O'Donnell et al., 2011). Therefore, caspase-8 prevents necroptosis by inactivating the necroptosis machinery, following receptor stimulation.

1.6.4 Is there a role for mitochondria in necroptosis?

Because mitochondria play a central role in the induction of apoptosis, it is tempting to speculate that mitochondria may also regulate necroptosis. As early as 1992 the cytotoxic effects of TNF had been linked to mitochondrial reactive oxygen species (ROS) generation (Schulze-Osthoff et al., 1992). Indeed, ROS accumulation has been implicated in driving the necrotic phenotype downstream of RIPK1/RIPK3 activation (Cho et al., 2009; Festjens et al., 2007; Zhang et al., 2009). ROS scavenging, however, has failed to convincingly demonstrate that ROS is a conserved and essential effector of RIPK3-driven necrosis, suggesting it may be a consequence of necroptosis induction as opposed to a driver of it. In a pre-necrotic complex, RIPK3 has been shown to associate with multiple metabolic enzymes including glycogen phosphorylase (PYGL), glutamate ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1) which is thought to cause their deregulation

and contribute to ROS generation (Zhang et al., 2009). Furthermore, the RIPK1/RIPK3/MLKL necrosome has been proposed to activate phosphoglycerate mutase 5 (PGAM5) and Dynamin-related protein 1 (Drp-1) to cause mitochondrial fragmentation and cell death (Wang et al., 2012). Importantly however, cells entirely depleted of mitochondria by overexpression of Parkin, are not compromised in necroptosis induction, rendering mitochondrial involvement in necroptosis controversial (Tait et al., 2013).

1.7 THE ROLE OF RIPK1 IN NECROPTOSIS IS COMPLEX

Necroptosis is often considered, or even defined as a RIPK1- and RIPK3-dependant cell death (Cho et al., 2009; Ofengeim and Yuan, 2013). The requirement of RIPK1 for necroptosis is presumed due to the ability of Necrostatin-1, a RIPK1 kinase inhibitor, to block the process (Degterev et al., 2008; Duprez et al., 2011; Zheng et al., 2008). At odds with this definition, however, RIPK1 has been shown to be dispensible for necroptosis in several settings. Indeed, Vaux and colleagues have shown that TNF-induced necroptosis still occurs in MEFs derived from RIPK1 null animals (Moujalled et al., 2013). Furthermore, knockdown of RIPK1 potentiated TNF-induced necroptosis in L929 cells and did not block necroptosis of RAW macrophages treated with LPS and zVAD (He et al., 2011; Vanlangenakker et al., 2011). All available evidence would suggest that RIPK3 is absolutely required for necroptosis, however, it appears that RIPK1 activity should not be considered a defining feature of this cell death modality.

1.7.1 Genetic evidence indicates that RIPK1 suppresses necroptosis

RIPK1 knockout is embryonic lethal, likely due to enhanced apoptosis as a consequence of impeded RIPK1-dependent NFκB activation (Kelliher et al., 1998; Zhang et al., 2011). Caspase-8 or FADD knockout is lethal, but can be rescued by combined deletion of RIPK3, suggesting Caspase-8 restrains RIPK3-dependant necroptosis during development (Dillon et al., 2012; Kaiser et al., 2011; Oberst et al., 2011). Therefore, if necroptosis is also RIPK1 dependant, deletion of RIPK1 should rescue loss of caspase-8-mediated lethality. Indeed, loss of caspase-8 should also prevent excessive apoptosis as a result of RIPK1 loss in this context. Recent studies have adressed this issue, thus helping to elucidate the role of RIPK1

in necroptosis. Caspase-8 knockout fails to rescue the RIPK1 knockout phenotype (Dillon et al., 2014; Rickard et al., 2014). Moreover, loss of RIPK3 also fails to suppress lethality as a result of RIPK1 knockout (Dillon et al., 2014; Kaiser et al., 2014; Rickard et al., 2014). Rather surprisingly, Three recent reports suggest that combined loss of RIPK3 and Caspase-8 blocks the lethal effect of RIPK1 loss (Dillon et al., 2014; Kaiser et al., 2014; Rickard et al., 2014). This demonstrates that RIPK1 acts as a suppressor of caspase-8-dependant apoptosis as well as RIPK3-dependant necroptosis. These genetic models suggest that the definition of RIPK1 as a positive regulator of necroptosis requires further clarification.

1.7.2 The RIPK1 kinase inhibitor Necrostatin-1

Necrostatin-1 was discovered in a cell-based chemical library screen for inhibitors of caspase-independent TNF-driven cell death (Degterev et al., 2005). While the original Necrostatin-1 molecule, methyl-thiohydantoin-Trp (MTH-Trp) could inhibit IDO and RIPK1, an improved analogue of Necrostatin-1, 7-Cl-O-Nec-1 (5-(7-chloro-1H- indol-3-yl)methyl)-3-methylimidazolidine-2,4-dione), was developed that specifically inhibits the kinase activity of RIPK1. Necrostatin-1 binds to a hydrophobic pocket between the N-lobe and C-lobe of the RIP1 kinase domain, which locks RIPK1 in an inactive conformation (Xie et al., 2013). Most kinase inhibitors target the ATP site of the kinase in its active Asp-Phe-Gly-in state. Necrostatin-1, however, binds a pocket adjacent to the ATP-binding site, trapping the kinase in an inactive, Asp-Phe-Gly-out state. Indeed, Necrostatin-1 inhibits RIPK1 1,000 times more efficiently than 485 other human kinases, including other RIP family members (Ofengeim and Yuan, 2013). Furthermore, Necrostatin-1 also targets RIPK1 *in vivo*, as it fails to inhibit pathology in the absence of RIPK1 (Degterev et al., 2008).

In the absence of specific markers for necroptosis *in vivo*, Necrostatin-1 has often been used to define a pathological state as necroptosis-dependent. Indeed, Necrostatin-1 affords protection from the lethal effects of TNF-induced SIRS in mice (Duprez et al., 2011). Similarly, Necrostatin-1 ameliorated symptoms of Huntington's disease, that is thought to proceed by deregulated neuronal cell death (Zhu et al., 2011). Necrostatin-1 also prevented renal failure in a mouse model of

ischemic reperfusion (Linkermann et al., 2012b). As described above, however, RIPK1 appears to be dispensable for necroptosis *in vivo*, and may in fact serve to heighten the necroptotic threshold. Importantly, Necrostatin-1 appears to block necroptosis in all cases, even when RIPK1 appears to be dispensable for the process (Kearney et al., 2014; Vanlangenakker et al., 2011). This discrepancy however, has remained unexplained and suggests that our understanding of the mechanism by which RIPK1 and Necrostatin-1 regulate necroptosis is incomplete.

1.8 NECROSIS AND NECROPTOSIS AS A TRIGGER FOR INFLAMMATION

Necrotic cell death is known to cause an inflammatory response in the absence of infection (Matzinger, 1994). However, exactly how necrotic cell death promotes inflammation remains relatively obscure. Sterile inflammation is thought to occur as a result of the release of particular cell constituents called 'danger signals' or DAMPs (Chen and Nunez, 2010; Matzinger, 1994) (Figure 1.10). DAMPs are thought to be endogenous molecules with intrinsic inflammatory potential, similarly to cytokines. Several endogenous DAMPs have been proposed, including DNA, ATP, histones, CIRP and HMGB1, however, the relative potency of these molecules in comparison to conventional cytokines remains unknown. Because necrosis is generally pro-inflammatory, in comparison with apoptosis, it is widely presumed that the switching of TNF-induced signaling to necroptosis exacerbates inflammation in this setting (Kaczmarek et al., 2013). This is presumed because unregulated necrosis promotes the release of pro-inflammatory DAMPs, which must also occur during necroptosis (Kaczmarek et al., 2013; Moriwaki and Chan, 2013). While this presumption may seem legitimate, few studies have directly analyzed the effects of necroptotic DAMP release on inflammation *in vivo*. Perhaps the best evidence that necroptosis drives sterile inflammation comes from data generated from conditional RIPK1 knockout in epithelial tissue. Mice lacking epithelial RIPK1 developed severe skin inflammation, which was prevented by the absence of RIPK3, suggesting necroptosis was indeed responsible for the pathology (Dannappel et al., 2014). However, an alternative explanation is that RIPK3 can also promote cytokine production to drive inflammation. Similarly, increased circulating levels of the cytokine DAMP IL-33 can be detected in RIPK1 null mice, which is prevented by RIPK3 or MLKL loss (Rickard et al., 2014). It is important to note however, that

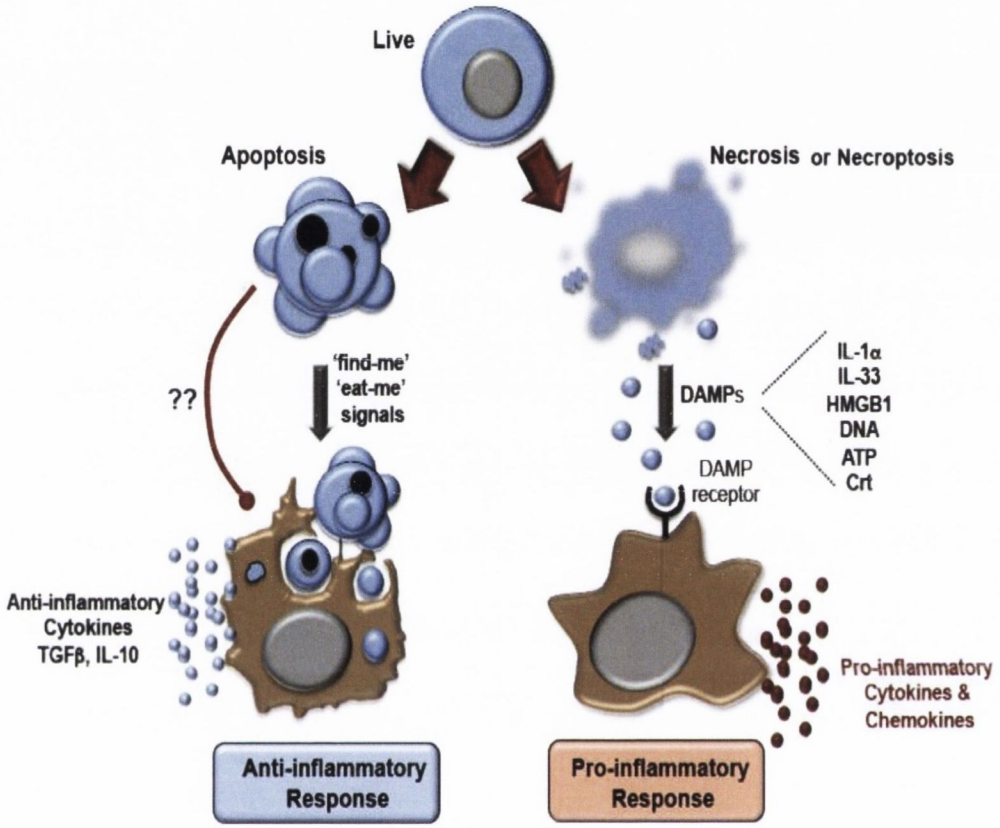

Figure 1.10

Inflammatory outcomes of apoptosis versus necrosis

Apoptosis is thought to represent a cell death mode that avoids explicit activation of the immune system. Apoptotic cells display molecules on their surface, such as phosphatidylserine, that facilitates their recognition and engulfment by phagocytes. Phagocytic cells then release anti-inflammatory cytokines such as IL-10 and TGFβ. In contrast, necrosis or necroptosis is thought to promote inflammation by release of particular cell constituents called DAMPs, which can activate innate immune cells. Perhaps the best candidates for DAMPs are conventional cytokines such as IL-1 and IL-33, which have inflammatory potential similar to TNF.

genetic alterations leading to RIPK3-dependant cell death *in vivo* often involve break-down of barrier tissues such as epthithelium and endothelium. It is difficult to separate, therefore, the inflammatory contribution of endogenous DAMPs from excessive microbial entry into damaged barrier tissue.

1.9 IS NECOPTOSIS A STRATEGY TO COMBAT PATHOGENS?

Necroptosis is thought to be a host strategy to combat replication of viruses and intracellular pathogens (Cho et al., 2009; Mocarski et al., 2012). Several investigators have suggested that viruses may inadvertently trigger necroptosis, thereby leading to rapid cell death and termination of virus replication (Mocarski et al., 2012). Indeed, RIPK3 knockout mice are susceptible to Vaccinia virus infection, suggesting RIPK3-dependant necroptosis is used to control viral spread (Cho et al., 2009). In support of this concept, murine cytomegalovirus (CMV) expresses vIRA, which disrupts the RIPK1/RIPK3 interaction to suppress both CMV- and TNF-induced necroptosis (Upton et al., 2010). Importantly, control of vIRA mutant virus in wild type mice is normalized in RIPK3-deficient mice, validating inhibition of RIPK3 activity as a viral strategy. Necroptosis, however, does not always appear to be detrimental to pathogens. Certain viruses express CrmA, a potent inhibitor of Caspase-8 and Caspase-1. Virus deficient in CrmA is less pathogenic than wild type virus, that may use CrmA to trigger necroptosis (MacNeill et al., 2009). Necroptosis may be beneficial to pathogens by terminating pro-inflammatory cytokine production by responder cells, following pathogen detection or TNF stimulation. In support of this concept, Infection with the intracellular bacterium *Salmonella Typhimurium* has also been shown to lead to RIPK3-dependent necroptosis (Robinson et al., 2012). Infection of RIPK3 null macrophages (incapable of undergoing necroptosis), however, led to decreased bacterial burdens, suggesting that rapid RIPK3-dependent necroptosis of macrophages is beneficial to the pathogen and helps to attenuate the host immune response. Furthermore, *Listeria monocytogenes* triggers IRF-3-dependant macrophage necroptosis which leads to increased bacterial burden (Di Paolo et al., 2013). Therefore, evidence would suggest that the consequences of necroptosis for pathogen replication is context dependent, with necroptosis being detrimental to some viruses, but aiding the pathogenesis of particular bacteria, perhaps by

terminating inflammatory responses.

1.10 CONCLUSIONS

Cell death is a normal and important cellular process in the body. Apoptosis is a programmed mode of cell death which prevents damage to surrounding tissue and restrains activation of the immune system. Necrosis is a non-programmed cell death, in which cellular constituents are liberated and are thought to promote activation of the immune system and inflammation. Indeed, innate immunity and inflammation is often initiated by release of TNF, particularly by macrophages that respond to necrotic cell death or microbial products. Subsequently, TNF drives inflammation through the production of multiple downstream cytokines and chemokines that collectively shape immune responses.

TNF signaling cascades are complex but are now relatively well understood. RIPK1 and IAPs function to promote TNF-induced inflammation, while restraining TNF-induced cell death. Furthermore, caspase-8 prevents TNF from inducing a programmed necrotic cell death, called necroptosis, which is thought to promote inflammation by release of DAMPs. Therefore, TNF signaling is highly regulated, as divergent outcomes of TNF stimulation have dramatic consequences for innate immunity and inflammation. Understanding the molecular control and immunological consequences of diverse TNF-induced outcomes is of extreme importance, as deregulated TNF signaling is a powerful driver of multiple pathologies.

1.10.1 Thesis aims

IAPs have been intensively studied in relation to their ability to repress cell death signals, particularly TNF-induced apoptosis. However, how IAPs regulate production of TNF-induced cytokines remains unknown. As IAPs can regulate NFkB activation, we ask; do IAPs modulate TNF-induced inflammation? Necroptosis is triggered by ligands that are intrinsically pro-inflammatory, such as TNF and LPS. Therefore we ask; does switching ligand-induced signaling to necroptosis diminish the inflammatory outcome by terminating pro-inflammatory cytokine and chemokine production? Finally, necroptosis is often defined as a RIPK1 kinase- and RIPK3 kinase-dependent cell death. Here, we explore whether RIPK1 functions as a

positive or negative regulator of necroptosis and whether inhibition of RIPK1 kinase activity blocks necroptosis in the way this is interpreted at present.

CHAPTER 2

MATERIALS AND METHODS

2.1 GENERAL REAGENTS

Recombinant TNFα was purchased from Roche (UK) and from eBiosciences (UK). The IAP antagonist BV6 was from Genentech and the peptide caspase inhibitor zVAD-fmk was from Bachem (UK). The following inhibitors were obtained from commercial sources: UO126 and PD98059 was from Cell Signaling (UK), BAY117082, ERK peptide, SP600125 and SB202190 were from Calbiochem (UK). SB203580 was from Invivogen (UK). Necrostatin-1 was from Sigma (Ireland). Unless otherwise indicated, all other reagents were purchased from Sigma Chemical Co. (Ireland).

2.2 ANTIBODIES

Specific antibodies used in these studies were obtained from commercial sources as follows: anti-XIAP, anti-IKBα , anti-RIPK1 and anti-IKKα were from BD (UK). Anti-cIAP-1, anti-cIAP-2, and anti-TNFα neutralising antibody were from R&D Systems (UK). Anti-phospho IKKα/β, anti-MEK, anti-phospho MEK, anti-ERK, anti-phospho ERK, anti-p38, anti-phospho p38, anti-JNK, anti-phospho JNK and anti-mouse IKBα were from Cell Signaling (UK). Anti NFκB p65 was from Santa Cruz Biotechnology (U.S.A). Anti mouse RIPK3 was from ProSci (UK). Anti-actin was from ImmunO (UK).

2.3 PURIFICATION OF NUCLEIC ACIDS

2.3.1 Baterial strains

E.Coli strain DH5α was routinely used to amplify plasmid DNA.

2.3.2 Preparation of competent bacteria

Bacteria were prepared to become competent for plasmid DNA transformation using the Inoue method (Inoue et al., 1990). Initially, DH5α bacteria were streaked out onto LB agar plates (10 g/L Tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar, all from BD). After 12-16 h, single colonies were used to inoculate 3ml cultures in LB liquid broth (10 g/L Tryptone, 5 g/L yeast extract, 10 g/L NaCl, all from BD). The following day, the 3ml cultures were diluted into 50 ml of SOB medium (2% tryptone (BD), 0.5% yeast extract (BD), 10mM NaCL, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, to an OD_{600} of 0.1 AU. The cultures were then incubated at room temperature until an OD_{600} of 0.5 AU was reached. The Bacteria were then harvested by cenrtigugation at 2,500 g for 10 minutes. Bacteria were then resuspended in 20 ml of transformation buffer (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCL, pH 6.7, then incubated for 10 minutes on ice. Bacteria were again centrigugated and resuspended in 4 ml transformation buffer. DMSO was then added to a final concentration of 7%, and bacteria incubated on ice for 10 minutes. Bacteria were aliquoted and stored at -70 °C.

2.3.3 Transformation of competent bacteria

Plasmid DNA was introduced into competent bacteria by the heat shock method. Frozen aliquots of bacrteria were thawed on ice and 50 µl added to a 13 ml bug culture tube. 1 µl of plasmid was added to the bacteria, mixed, and incubated on ice for 30 minutes. Bacteria were then heat shocked for 40 seconds at 42 °C, then incubated on ice for 2 minutes. 450 µl of LB medium was then added to the bacteria, followed by incubation at 220rpm in a shaker set to 37 °C for 1 hour. 20 µl was then spread on a LB agar plate with the appropriate antibiotic, and incubated at 37 °C overnight.

2.3.4 Plasmid Purification

(i) Plasmid miniprep

Single colonies from a plate of transformed bacteria were inoculated into 3 ml of LB liquid medium containing the appropriate antibiotic, and incubated overnight at 260

rpm at 37 ° C. 1 ml of each culture was then pelleted at 20,000 g for 1 minute in a microfuge. Bacterial pellets were resuspended in 100 µl of Buffer P1 (50mM Tris-HCL, 10 mM EDTA, pH 8) then bacteria were lysed in 200 µl of buffer P2 (200 mM NaOH, 1% SDS) for 1 minute on ice. 200 µl of buffer P3 was then added (3 M KAc, pH 5.5) and incubated on ice for 3-5 minutes to allow precipitation of genomic DNA. Precipitated genomic DNA was then pelleted by centrifuging at 20,000 g for 15 minutes, and the supernatant containing plasmid DNA was isolated. Plasmid DNA was then precipitated by addition of 0.7 X volumes of isopropanol and incubated at room temperature for 20 minutes. Plasmid DNA was pelleted at 20,000 g for 10 minutes, then washed with 1 ml of 70% ethanol. Centrigugation was repeated and the pellet air dried for 10 minutes. DNA was re-suspended in 20 µl of 0.5 x TE (10 mM Tris-HCL, 1 mM EDTA, pH 8). 1-2 µl of purified plasmid DNA was then electrophoresed on 0.8 % agarose gels, stained with ethidium bromide and viewed in a UV gel dock.

(ii) Plasmid midiprep

50 ml of LB liquid medium was inoculated with 50 µl of a 3 ml bacterial culture which was produced from a transformed bacterial agar plate, as described. The culture was then grown overnight at 37 ° C, 260 rpm. The next day, the culture was pelleted at 5000 g for 10 minutes and resuspended in 4 ml of buffer P1. Bacteria were lysed by addition of 4 ml buffer P2, then incubated on ice for 1 minute. 4 ml of buffer p3 was then added, and incubated on ice for 3-5 minutes to facilitate precipitation of genomic DNA. Precipated DNA was pelleted by centrifugation at 15,000 g for 15 minutes, and the plasmid containing supernatant isolated. Supernatant was then allowed to flow through a Qiagen Q100 column, which was first equilibrated with 4 ml of Qiagen QBT buffer (750 mM NaCl, 50 mM MOPS, pH 7, 15% isopropanol, 0.15% Triton X-100). Column bound DNA was then washed with 2 x 10 ml of Qiagen buffer QC (1 M NaCl, 50 mM MOPS, pH 7, 15% isopropanol), then DNA was eluted from the column with 5 ml of Qiagen buffer QF (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15% isopropanol). Plasmid DNA was then precipitated by addition of 0.7 volumes (3.5 ml) of isopropanol and incubating for 20 minutes at room temperature. Precipitated DNA was pelleted at 20,000 g for 20

minutes, washed with 2 x 1 ml of 70% ethanol, then again pelleted at 20,000 g for 10 minutes. The DNA pellet was resuspended in 200 µl of 0.5 x TE.

2.3.5 Agarose gel electrophoresis

DNA yield and purity was typically analysed by electrophoresis through agarose gels alongside a ladder of DNA fragments of known molecular weights. Gels were prepared as 0.6-1.0 % agarose in TAE (40 mM Tris-HCl, 1 mM EDTA, pH 8). The agarose suspension was melted by microwaving, then allowed to set in casting rigs. DNA of interest was diluted in loading buffer and electrophoresed through the gel at 75 V. Gels were incubated with ethidium bromide for 20 minutes, then visualized in a Biorad Gel Doc 2000 UV system.

2.3.6 Quantification of DNA

DNA was routinely quantified by spectrophotometry. DNA was diluted 1:40 in dd-H_2O, then $OD_{260/280}$ was measured in a quartz cuvette in a Biorad spectrophotometer. DNA concentration (ng/µl) was then calculated using the following equation. DNA Concentration = $(OD_{260/280})(2)$(dilution factor)(50).

2.3.7 Genecleaning procedure

As a prerequisite to cloning, genecleaning was conducted to ensure DNA was free from any contaminants which may interfere with later reactions. DNA was first resolved by electrophoresis and the band of interest was excised from the gel under a UV lamp. Agarose was then melted with 3 x volumes of 6 M NaI, then 5 µl/µg DNA of silica (100 mg/ml suspension in 3 M NaI) and rotated for 10 minutes at room temperature to allow DNA binding to silica. DNA bound to silica was pelleted at 20,000 g for 5 seconds, then washed in 5 x 500 µl volumes of wash buffer (10 mM Tris-Hcl, 50 mM NaCl, 2.5 mM EDTA, pH 7.5, 50% ethanol. The DNA pellet was the air dried for 10 minutes and resuspended in dd-H_2O.

2.3.8 Restriction digests.

DNA was digested using restriction endonucleases, using manufactures instructions. Typically, DNA was incubated with single restriction enzymes, or in combination, then incubated overnight at 37 ° C. DNA was then electrophoresed and restriction fragments viewed in a UV gel doc. Restriction digests were routinely used to confirm the identity of received plasmids.

2.3.9 DNA ligation reactions

To clone particular genes, DNA fragments were ligated after restriction digest and genecleaning procedures. Typically, 200 ng of vector was incubated with a 2:1 molar ratio of insert DNA. The reaction was brought to 10 µl with dd-H_2O, and incubated at 45 ° C, to melt re-annealed DNA ends. T4 DNA ligase was then added in the presence of T4 ligase buffer, then incubated overnight at 16 ° C. 2 µl of reaction was then transformed into competent DH5α, and resulting colonies screened for insert uptake.

2.3.10 Polymerase Chain Reaction (PCR)

PCR reactions were conducted to amplify genes for cloning, and to confirm insert entry into vectors. PCR reactions were carried out on a 100 µl scale. Taq DNA polymerase was obtained from Qiagen and was used at 2.5 units per reaction. Taq was added to DNA template (100 ng) in the presence of the following constituents:

- Taq buffer from Qiagen
- 200 µM dATP, dTTP, dCTP, dGTP
- 100 nM of forward and reverse primers

The reaction mix was then subject to the following PCR programme:

Denaturing: 3 minutes at 94 ° C

30-35 cycles of: Denaturing - 1 minute at 94 ° C

Annealing - 1 minute at 55-60 ° C

Elongation - 1 minute per kB DNA at 72 ° C

Final elongation: 10 minutes at 72 ° C

PCR was then confirmed by electrophoresing 1-2 µl of reaction mix on an agarose gel, followed by ethidium bromide staining and viewing in a UV gel doc.

2.4 PROTEIN ANALYSIS

2.4.1 SDS-PAGE electrophoresis and immunoblotting

To analyse proteins, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was typically utilised. Protein samples were initially prepared in SDS-PAGE sample buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 10& glycerol, 2.5% β-mercaqptoethanol), then boiled for 7 minutes to denature proteins. Saples were then loaded into 8-12% polyacrylamide gels and electrophoresed at 55 V through the stacking gel and 75 V through the resolving gel. Resolved proteins in SDS-PAGE gels were then transferred on to nitrocellulose membranes overnight at 40 mA. Membranes were then blocked for 1 h in blocking buffer (5 % non- fat- dried milk, 0.05% NaN_3 in Tris buffered saline, Tween-20 (TBST)). Membranes were then probed with primary antibody, typically diluted 1:1000 in blocking buffer for 2 h at room temperature. If proteins were difficult to detect, membranes were incubated overnight at 4 ° C. Membranes were then washed for 3 x 10 minutes with TBST, followed by incubation with the appropriate secondary antibody conjugated to HRP, diluted 1:1000 in TBST containing 5% NFDM. After a final round of 3 x 10 minute washes, proteins were detected by addition of a West Coast Super Signal (Thermo).

To analayse proteins under non-reducing conditions (which facilitates detection of protein oligomers), β-mercaqptoethanol was omitted from the SDS-PAGE sample buffer. All other aspects of the procedure were then carried out as described above.

2.4.2 Detection of cytokines and chemokines by ELISA

Detection of cytokines and chemokines from cell culture supernatants or *in vivo* lavage fluid was carried out using commercially available paired ELISA antibodies as follows: Human TNF, human MCP-1, human CXCL1, human sICAM-1, human RANTES human GM-CSF, mouse G-CSF, mouse M-CSF, mouse KC, mouse IL1β

and mouse IL1α were from R&D systems (UK). Human IL-6, human IL-8, mouse IL-6 and mouse MCP-1 were from eBiosciences (UK). Typically 50 µl of capture antibody per well was coated on a 96 well plate according to the manufacturers dilution factor, and incubated overnight at room temperature. Wells were then washed x 3 with 150 µl of wash buffer (0.05% Tween-20 in PBS, pH 7.2), then blocked for 1 h in sample buffer (1% BSA in PBS, pH 7.2). Samples were diluted as required in sample buffer, then 50 µl of sample was added per well and incubated for 2 h at room temperature. Wells were washed again x 3, then 50 µl of secondary antibody diluted appropriately, was added to each well for 2 hours. Wells were then washed again x 3 and 50 µl of streptavidin-HRP (R&D systems) added and incubated for 30 minutes at room temperature. Finally, wells were washed again x 3 prior to addition of 50 µl TMB substrate solution. The reaction was stopped with 30 µl of 3 N sulphuric acid, the absorbance read on an ELISA plate reader. Each assay was repeated a minimum of three times and all cytokine assays were carried out using triplicate samples from each culture.

2.6 *IN VITRO* CELL CULTURE

2.6.1 Cell culture conditions

Cell cultures were maintained at 37° C in 5% CO_2. The following media requirements were applied to the cell lines listed below: HeLa and L929 cells were grown in RPMI supplemented with 5% FCS. HaCaT, HT-29, MEFs, RAWs and BMDMs were grown in DMEM supplemented with 10% FCS. *In vivo*-derived peritoneal exudates were incubated in RPMI supplemented with 10% FCS. Primary HUVEC cells were cultured in endothelial cell growth media with added growth supplement (PromoCell, Germany). Cells were passaged every 2-3 days. To passage adherent cell lines (HeLa, HT-29, HaCaT, MEFs, L929), cells were removed with Trypsin-EDTA (0.25% trypsin, 1 mM EDTA in HBSS). To inactivate the trypsin, fresh medium was added, then cultures spun down at 250 g for 5 mins, followed by resuspension in fresh medium and transferred to fresh plates. BMDMs were gently scraped into suspension, then diluted into fresh medium and re-plated.

2.6.2 Transient transfection of plasmid DNA

Genejuice (Merck) was used to transiently introduce DNA into HeLa cells. Cells were seeded at 1-2x10^5 in 6 well plates the day prior to transfection. 2 µl of Genejuice per well was then diluted to 100 µl with RPMI, mixed and incubated for 15 minutes. Diluted Genejuice was then added I a dropwise fashion onto 50 ng-1 µg of plasmid DNA in a 96 well plate and incubated for 20 minutes. Genejuice/DNA complexes were then added to cell monolayers and allowed 24-48 h for protein expression. The following CMV promotor- driven constructs were used: pRK.FLAG.RIPK1, pEBB.Ha.cIAP1, pEBB.Ha.cIAP2, pEBB.Ha.XIAP. Typically a GFP expressing cDNA (pAAV-GFP) was co-transfected to assess efficiency of the transfection on a given day

2.6.3 Transfection of siRNAs using electroporation

Nucleofection (Amaxa) was used to ablate expression RIPK1, RIPK3, MLKL, cIAP1, cIAP2 and XIAP in all cell lines used. Typically, 5x10^5 per siRNA condition were pelleted at 250 x g for 5 mins, then resuspended in 100 µl of nucleofection buffer A. Suspended cells were then mixed with 10 µl of 20 µM siRNA, transferred to cuvettes and electroporated on the suitable programme. Cells were then diluted into culture medium and seeded at the required density in 6 or 24 well plates. After 48 hours, cells were treated as indicated. A fraction of the cells were lysed in SDS-PAGE sample buffer 48 h post nucleofection, to analyse knock down efficiency by immunoblotting. The following siRNA sequences were used:

cIAP-1#1: 5'-ggcaaaugcugcggccaaca-3',

cIAP-1#2: 5'-uucguacauuucucucuuua-3',

cIAP-2#1: 5'-guucaagccaguuacccuc-3',

cIAP-2#2: 5'-aagugguagggacuugugc-3',

XIAP-1#1: 5'-gugguaguccuguuucagc-3',

XIAP-1#2: 5'-gcaguugacaaguguccca-3',

RIPK-1#1: 5'-ggagcaaacugaauaaugaagagca-3',

RIPK-1#2: 5'-ggagcaaacugaauaaugaagagca-3',

mouse RIPK1#1: 5'-ccacuagucugacugauga-3',

mouse RIPK1#2: 5'-gaggauauucucaggcuucagguccuu-3',

mouse RIPK3#1: 5'-cccgacgaugucuucugucaa-3',

mouse RIPK3#2: 5'-aagauuaaccauagccuucaccuccca-3',

mouse MLKL#1: 5'-gagauccaguucaacgaua-3',

mouse MLKL#2: sense: 5'-ggaauaccguuucagaugu-3'.

2.6.4 Light and UV microscopy

An Olympus IX71 light microscope with an integrated camera, was used to aquire phase contrast images, typically at 100x magnification. For analysis of UV fluorescence, the appropriate filter in combination with a UV burner was utilised on the Olympus IX71 microscope.

2.7 ANALYSIS OF CELL DEATH

2.7.1 Apoptosis assays

Typically, cells were plated at 2 x 10^5 cells/well in 6-well plates and treated as indicated 24 h later. Apoptosis was enumerated based on cell morphology (cell rounding, detachment from the plate, nuclear condensation and presence of apoptotic bodies) and by flow cytometry-based Annexin V/PI staining). To assess apoptosis as a result of plasmid transfection, GFP was co-transfected with the cDNA of interest, and percentage of GFP positive cells that displayed apoptotic morphology was counted as described above. A minimum of 300 cells was counted in each treatment. Each assay was repeated a minimum of three times.

2.7.2 Necroptosis assays

Typically, cells were plated at 2 x 10^5 cells/well in 6-well plates and treated as indicated 24 h later. Necroptosis was enumerated based on cell morphology (cell swelling, detachment from the plate, membrane rupture) and by flow cytometry-based Annexin V/PI staining). A minimum of 300 cells was counted in each treatment. Each assay was repeated a minimum of three times.

2.7.3 Crystal violet staining

To analyse cell survival by crystal violet staining, medium was gently removed from culture dishes and monolayers washed with 1 ml PBS. 1 ml of crystal violet stain (0.5% crystal violet, 25% methanol) was then added to monolayers and incubated for 5 minutes. Plates were then gently washed with water, then digital images obtained for analyses.

2.7.4 Analysis of cell death by flow cytometry

To quantitate apoptosis or necroptosis, cell culture samples were added to an equal volume of 2 x annexin binding buffer (20 mM HEPES-NaOH, 300 mM NaCl, 10 mM KCl, 2 mM $mgCl_2$, 3.6 mM $CaCl_2$, 2 µg/ml annexinV-FITC), containing propidium iodide at 20 µg/ml. Cell fluorescence was then analysed on a flow cytometer (FACScalibur, BD), using CellQuest software.

2.8 CHEMOTAXIS ASSAYS

Chemotaxis assays were performed in Neuro Probe 10-Well Chemotaxis Chambers as per the manufacturer's instructions. Supernatants (150 µl) were added to the bottom well of a chemotaxis chamber, and then 8 µM nitrocellulose filters were placed on top and secured by application of the top chamber, to which 200 µl of THP-1 cells or peritoneal exudate cells were added at $1x10^6$/ml. Total cells entering

the bottom chamber were counted after 6 h. For antibody-mediated depletion of chemokines, supernatants were incubated with protein A/G agarose coupled to specific antibodies (1–2 mg/ml) for 2 hr, followed by depletion of the agarose-antibody-chemokine complexes from the supernatants by centrifugation. Depletion of specific chemokines was then confirmed by ELISA.

2.9 *IN VIVO* ANALYSIS OF INNATE IMMUNE REACTIONS

Wild type C57BL/6 or BALB/c mice were intraperitoneally injected with the indicated treatments. After 24 h, mice were sacrificed and the peritoneal cavity was washed with 4-5 ml of PBS. The fluid containing innate immune cells was then retrieved for analysis. Typically, total cellularity counts were performed in triplicate, excluding erythrocytes. For splenic cellularity counts, samples were first incubated for 5 minutes with RBC lysis buffer, then spun down at 200 x g for 5 mins and resuspended in PBS. Cells were then immunostained with primary antibody for 1 h on ice, followed by staining with secondary antibody conjugated to a phlorophore. Populations were then analysed on a flow cytometer (FACScalibur, BD). All animals were purchased from Harlan (UK). Animal experiments were in accordance with the regulations of the Trinity College Dublin ethics committee and the Irish Department of Health.

CHAPTER 3

IAPS AND THEIR ANTAGONISTS REGULATE SPONTANEOUS AND TNF-INDUCED CYTOKINE AND CHEMOKINE PRODUCTION

3.1 INTRODUCTION

IAPs are a diverse family of proteins that have been implicated as regulators of apoptosis, mitosis and inflammation (Gyrd-Hansen and Meier, 2010; O'Riordan et al., 2008; Silke and Brink, 2010). Indeed, it is becoming increasingly apparent that the original designation of this protein family as inhibitors of apoptosis is overly simplistic and evidence is accumulating to suggest that the IAPs implicated in cell death control, cIAP-1, cIAP-2 and XIAP, also play important roles as regulators of signal transduction events that promote inflammation (Damgaard and Gyrd-Hansen, 2011). The overwhelming majority of studies to date, however, have focused on the ability of these proteins to modulate apoptosis, particularly in the context of TNF receptor engagement, and few studies have explored the role of the IAPs in the production of pro-inflammatory mediators. Because TNF is a major driver of inflammation in response to infection, as well as in the context of inflammatory diseases (Brennan and McInnes, 2008; Feldman, 2008), the role of IAPs in shaping TNF-dependent inflammatory signaling is an important unresolved question.

Engagement of the TNF receptor with its cognate ligand can result in apoptosis via a caspase-8-dependent pathway that is now relatively well understood (Ashkenazi and Dixit, 1998). TNF-dependent apoptosis proceeds via recruitment of TRADD, FADD and caspase-8 to the cytoplasmic tails of trimerized TNF receptors, resulting in proximity-induced autoactivation of caspase-8 (Ashkenazi and Dixit, 1998). It is frequently overlooked, however, that the majority of cell types fail to undergo apoptosis in response to TNF receptor engagement, but can be sensitized through blocking NFκB activation signals (Beg and Baltimore, 1996; Van Antwerp et al., 1996). The identity of the NFκB-induced factor(s) affording protection from TNF-induced apoptosis have been the subject of debate, with cIAP-1, cIAP-2, A20 and FLIP emerging as candidates.

Recent evidence indicates that cIAP-1 and cIAP-2 play particularly influential roles in repressing TNFR-induced cell death signals. Several groups have independently reported that small molecule IAP antagonists can greatly sensitize cells towards

TNF-dependent apoptosis (Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). cIAP-1 and cIAP-2 are required for optimal TNF-induced canonical NFκB activation (Varfolomeev et al., 2008), most likely via their role as ubiquitin-ligases for RIPK1, a central participant in TNF-initiated signaling (Bertrand et al., 2008; Dynek et al., 2010). Antagonism of cIAP-1 and cIAP-2 activities, therefore, can sensitize towards TNF-induced apoptosis, apparently through suppression of cIAP-1/2-dependent RIPK1 ubiquitination, which is required for canonical NFκB activation (Bertrand et al., 2008; Varfolomeev et al., 2008). Recent studies also suggest that IAPs negatively regulate assembly of a RIPK1-containing complex 'the Ripoptosome', most likely through restraining recruitment of FADD and caspase-8 to RIPK1 (Feoktistova et al., 2011; Tenev et al., 2011).

As mentioned above, most cell types do not undergo apoptosis directly in response to TNF but require sensitization towards its pro-apoptotic effects through blocking NFκB activation. Independent of its ability to promote apoptosis, a key aspect of TNF function is its capacity to trigger the production of a battery of pro-inflammatory cytokines and chemokines, thereby initiating and/or escalating immune responses. Examples of TNF-induced cytokines and chemokines include IL-6, IL-8, GM-CSF and RANTES, which can instigate and amplify immune responses through triggering the acute phase response, recruitment of neutrophils and basophils to the site of inflammation and by eliciting increased production of monocytes/macrophages from bone marrow. TNF-induced cytokines have both powerful and diverse effects on the immune system and overproduction of TNF is frequently associated with autoimmune conditions such as rheumatoid arthritis and Crohn's disease (Feldmann, 2008). Given the key role of TNF as a major driver of inflammation in many settings (Brennan and McInnes, 2008; Feldman, 2008), agents that are capable of restraining TNF-induced cytokine production are of considerable therapeutic interest. Because IAP antagonism can dramatically alter sensitivity towards TNF-induced apoptosis, we wondered whether this could also modulate TNF-induced inflammatory cytokine production.

Here, we show that TNF-induced production of multiple pro-inflammatory cytokines

and chemokines can be attenuated through antagonizing IAPs. Knockdown experiments revealed that cIAP-2 and RIPK1 were required for optimal TNF-induced cytokine and chemokine production. Furthermore, a small molecule IAP antagonist also inhibited the production of multiple TNF-induced inflammatory mediators. However, in the absence of TNF stimulation, IAP antagonism alone led to spontaneous production of the chemokines, RANTES and IL-8, in certain cell types. These data suggest that strategies aimed at neutralizing IAPs to lower the threshold for apoptosis in cancer chemotherapy, may produce outcomes that relate to the ability of such compounds to influence inflammatory cytokine production, in addition to cell death. Thus, independent of their role as negative regulators of TNF-induced apoptosis, IAPs positively regulate the production of TNF-induced pro-inflammatory cytokines and chemokines and therefore act as important regulators of inflammation.

3.2 RESULTS

Several groups have independently demonstrated that small molecule IAP antagonists greatly sensitize many cell types to TNF-induced apoptosis (Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). However, because a major role of TNF *in vivo* is to promote the production of pro-inflammatory cytokines and chemokines in the context of innate immune responses, we wondered whether IAPs are also capable of influencing TNF-dependent inflammatory responses.

3.2.1 TNF induces the production of multiple cytokines in the absence of apoptosis

To explore the role of IAPs as regulators of inflammatory cytokine production, we initially used HeLa cells, which are insensitive to the apoptosis-inducing effects of TNF in common with numerous other cell types (Figure 3.1 A). However, HeLa cells can be sensitized to TNF-induced apoptosis through addition of transcriptional/translational inhibitors that block the actions of TNF-induced NFκB activation (Figure 3.1 A). Under conditions where TNF failed to induce apoptosis,

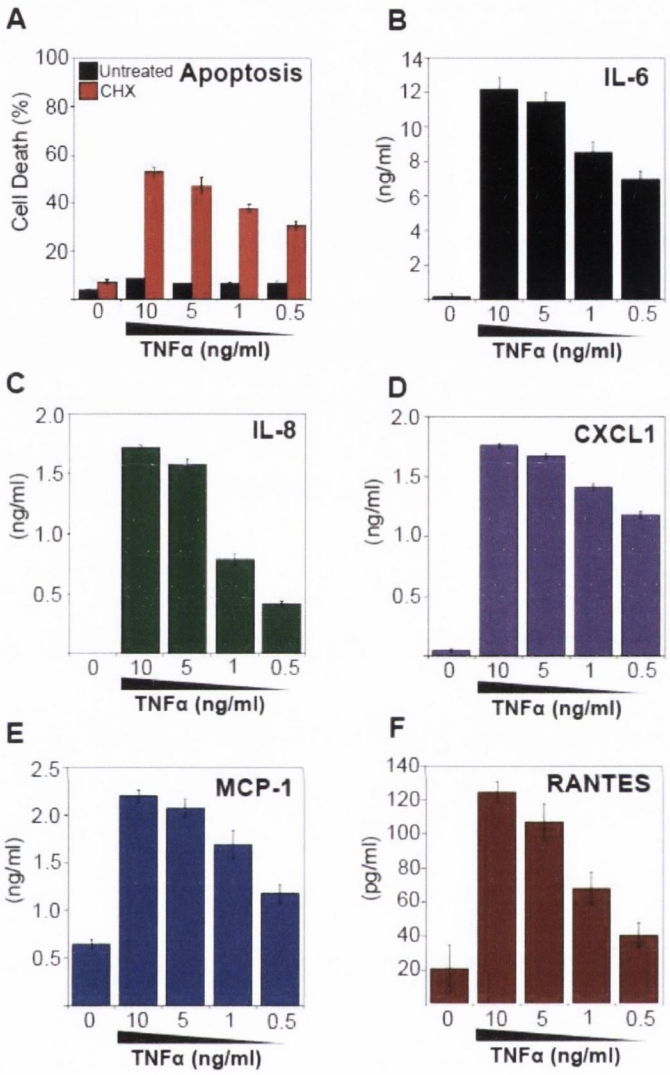

Figure 3.1

TNF induces secretion of multiple cytokines and chemokines. **(A)** HeLa cells were pre-treated or not with cycloheximide (CHX; 2 μM) for 2 h, followed by TNF stimulation at the indicated concentrations. After 24 h, apoptosis was determined by morphological assessment. Data represent triplicate counts of a minimum of 300 cells per treatment. **(B-F)** HeLa cells were stimulated with TNF at the indicated concentrations. After 24 h, cytokine concentrations in the culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

HeLa cells secreted large amounts of IL-6, IL-8, CXCL1, MCP-1 and RANTES in response to TNFR stimulation (Figure 3.1 B-F). Thus, independent of its ability to provoke apoptosis, TNF is capable of triggering the production of diverse pro-inflammatory cytokines and chemokines.

3.2.2 TNF-induced cytokine production can be inhibited through antagonism of IAPs

To explore the role of IAPs in TNF-induced cytokine and chemokine production, we used a bivalent small molecule IAP antagonist (BV6) that was previously shown to promote the rapid degradation of cIAP-1 and cIAP-2 (Varfolomeev et al., 2007). Consistent with this, BV6 promoted essentially complete degradation of cIAP-1, cIAP-2 as well as XIAP (Figure 3.2 A) and greatly sensitized HeLa cells to the apoptosis-inducing effects of TNF (Figure 3.2 B and C).

We then explored the impact of IAP antagonism on TNF-induced cytokine production. HeLa cells were stimulated with TNF in the presence or absence of IAP antagonist and, as before, this greatly sensitized towards TNF-induced apoptosis, which was completely suppressed through addition of the poly-caspase inhibitor zVAD-fmk (Figure 3.3 A). Importantly, TNF-induced production of IL-6, IL-8, CXCL1, MCP-1 and RANTES were profoundly inhibited through addition of IAP antagonist (Figure 3.3 B-F). This strongly suggests that IAPs are required for optimal TNF-induced cytokine production, consistent with previous observations that IAPs are required for canonical NFκB activation upon TNFR ligation (Mahoney et al., 2008). Because TNF induced widespread apoptosis in the presence of the IAP antagonist (Figure 3.3 A), it was possible that TNF-induced cytokine production was impaired merely as a consequence of cell death, leading to a decline in transcription/translation, rather than as a direct consequence of IAP antagonism. However, under conditions where TNF/BV6-initiated cell death was completely suppressed by zVAD-fmk (Figure 3.3 A), TNF-induced cytokine and chemokine production was still inhibited in the presence of BV6 (Figure 3.3 B-E). The exception to this was RANTES, the production of which was robustly increased

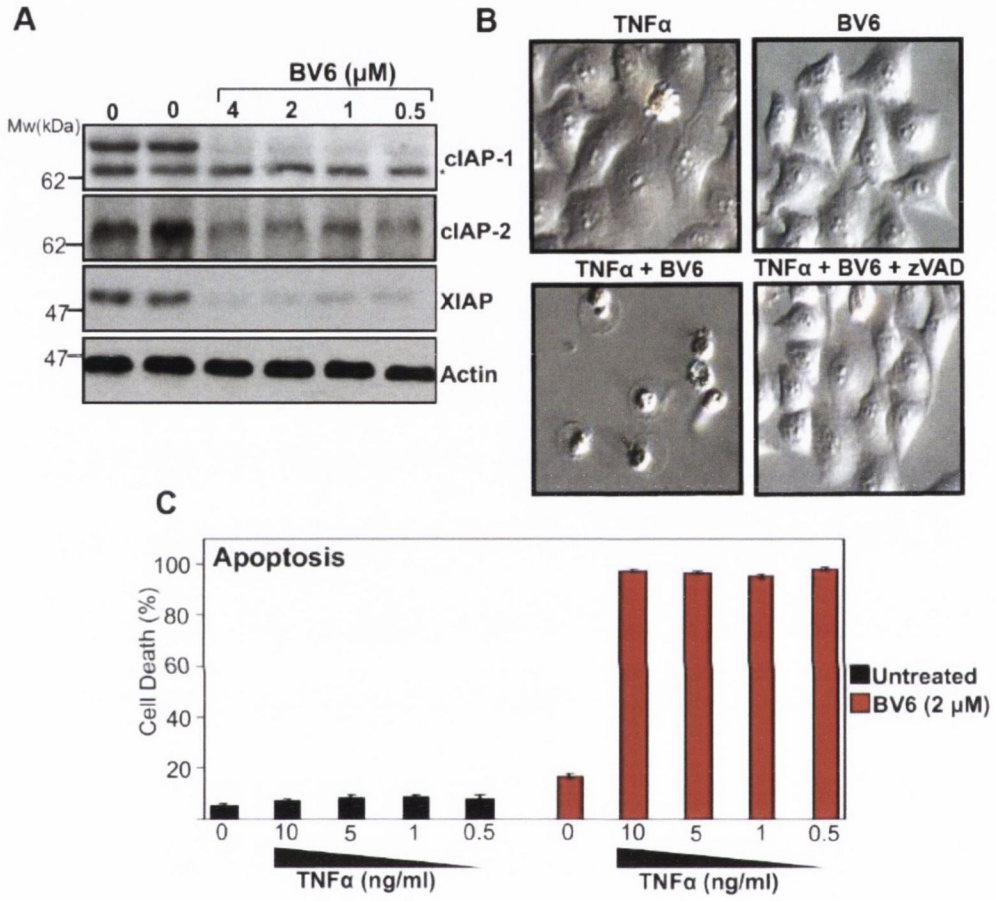

Figure 3.2

IAP antagonism sensitises towards TNF-induced apoptosis (A) HeLa cells were treated with BV6 at the indicated concentrations. After 24 h, levels of endogenous IAPs were analysed by immunoblotting. **(B)** HeLa cells were pre-treated for 2 h with BV6 (4 μM) in the presence or absence of zVAD-fmk (20 μM), followed by addition of TNF (10 ng/ml). After 24 h, cell cultures were visualized by phase-contrast microscopy. **(C)** Percentage apoptosis in cultures from **(B)** was determined by morphological assessment. Data represent triplicate counts of a minimum of 300 cells per treatment.

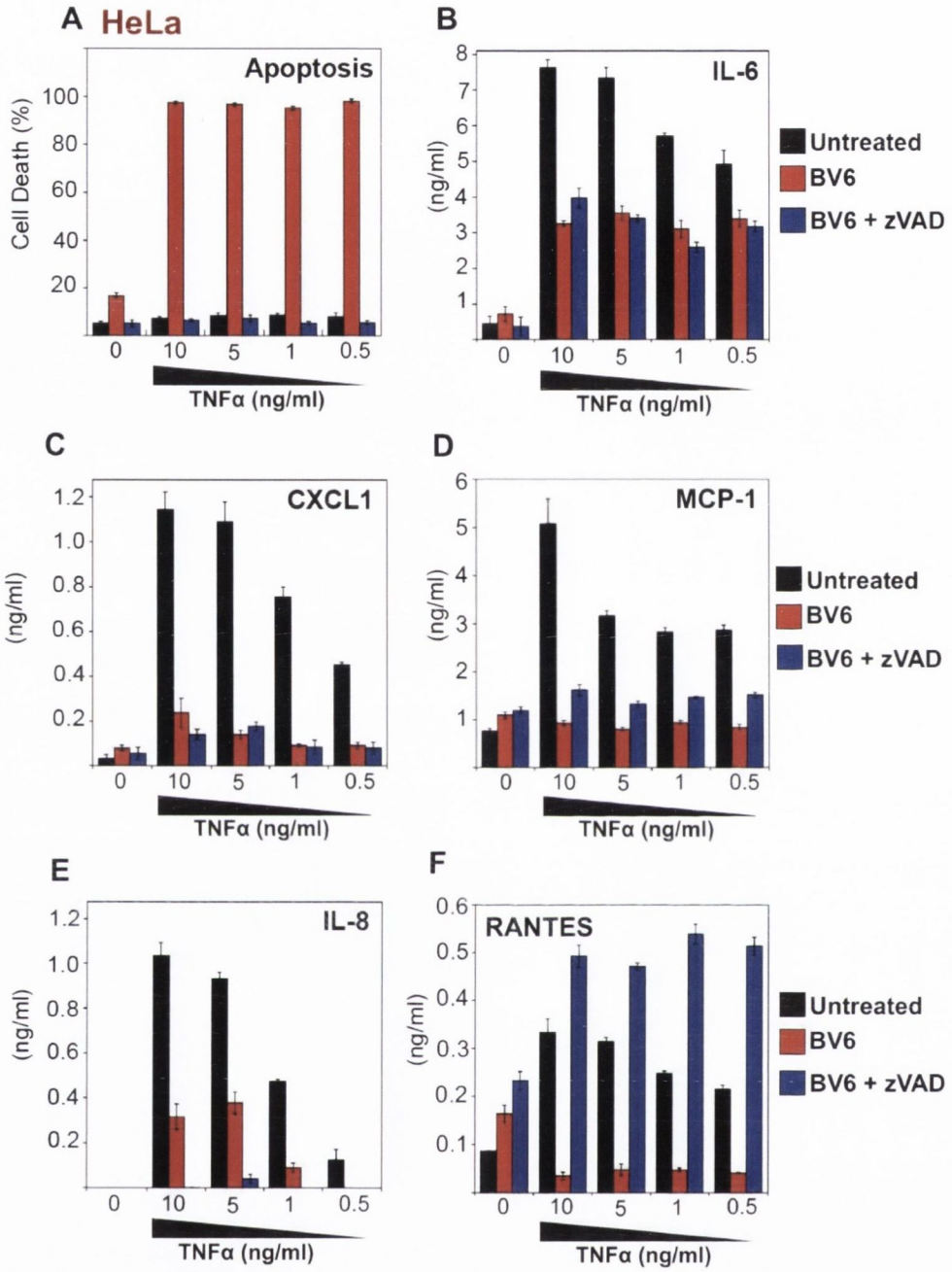

Figure 3.3

TNF-induced cytokine production is attenuated through inhibition of IAPs. (A) HeLa cells were either left untreated or treated with BV6 (4 μM) in the presence or absence of zVAD-fmk (20 μM) for 1 h, followed by stimulation with TNF at the indicated concentrations. After 24 h, apoptosis was determined by morphological assessment. A minimum of 300 cells were counted per treatment. (**B-F)** Cytokine concentrations in the culture supernatants from (*A*) were determined by ELISA. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

upon blocking cell death with zVAD-fmk (Figure 3.3 F). Indeed, the levels of this chemokine seen in response to TNF/BV6/zVAD-fmk treatment exceeded those seen in response to TNF alone (Figure 3.3 F). Indeed, it was possible that zVAD had direct inhibitory effects on TNF-induced signaling pathways leading to cytokine production. However, zVAD-fmk-treatment alone failed to influence TNF-induced IL-6, IL8, CXCL1 or RANTES, indicating that the suppression observed was indeed due to IAP loss (Figure 3.4 A-D).

3.2.3 TNF-induced cytokine production can be suppressed without sensitizing towards apoptosis

To further separate the effects of IAP antagonism on TNF-induced apoptosis versus TNF-induced cytokine production, we screened a panel of cell types for lack of sensitization to TNF-induced apoptosis in the presence of IAP antagonist. These experiments revealed that primary HUVECs fail to be sensitized towards the apoptosis-inducing effects of TNF through addition of BV6 (Figure 3.5 A and B). This enabled us to resolve whether inhibition of IAPs suppressed TNF-induced cytokine production completely independent of sensitization towards apoptosis. As Figure 3.6 *A-F* illustrates, BV6 inhibited TNF-induced production of IL-6, IL-8, GM-CSF, sICAM and RANTES in primary HUVECs, despite failing to sensitize these cells towards TNF-induced apoptosis under the same conditions. These data again demonstrste that IAPs are required for optimal TNF-induced pro-inflammatory cytokine and chemokine production and that synthetic IAP antagonists can influence this important aspect of TNF function.

3.2.4 IAP antagonism can lead to spontaneous production of chemokines

In contrast to the general suppression of TNF-induced cytokine and chemokine production observed in the presence of IAP antagonist, treatment of HeLa cells with BV6 alone led to spontaneous production of RANTES above basal levels (Figure 3.7 A). Indeed, RANTES production was further enhanced through inhibition of caspase activity in the presence of BV6 (Figure 3.7 A). We also observed a dramatic increase in RANTES release over a timecourse of BV6 treatment (Figure

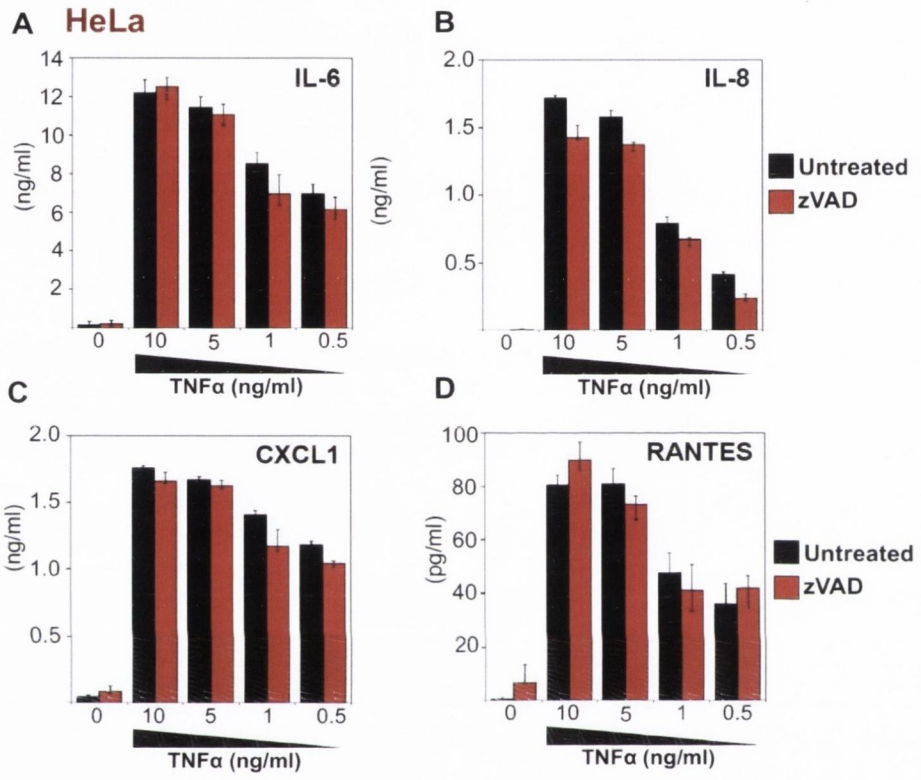

Figure 3.4

Caspase activity is not required for TNF-induced cytokine production. (A-D) HeLa cells were either left untreated, or treated with zVAD-fmk (20 μM) for 2 h, followed by stimulation with TNF at the indicated concentrations. After 24 h, cytokine concentrations in the culture supernatants were determined by ELISA. Results shown are representative of at least three independent experiments. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

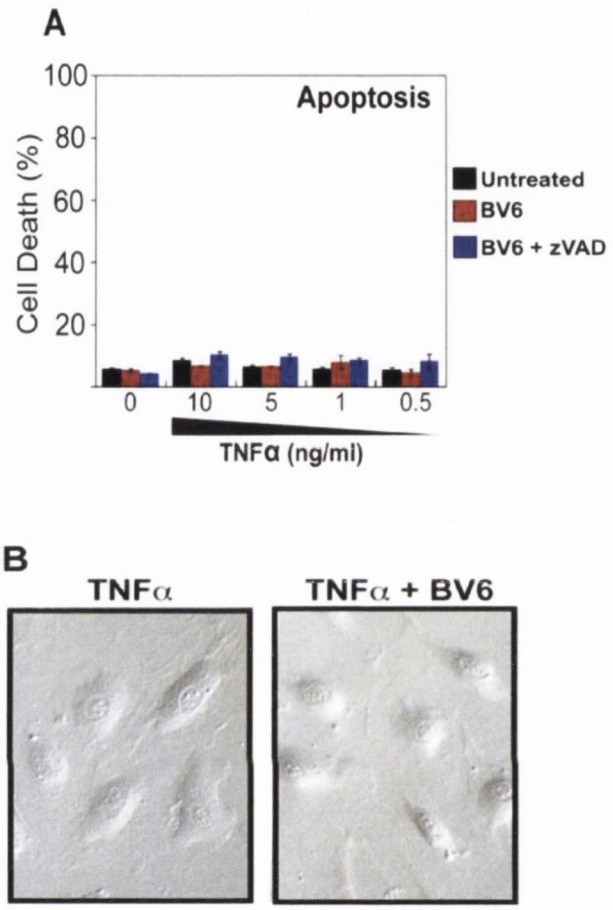

Figure 3.5

Primary HUVEC cells are resistant IAP antagonist / TNF- induced apoptosis. **(A)** Primary HUVEC cells were left untreated, or treated for 2 h with BV6 (4 μM) in the presence or absence of zVAD-fmk (20 μM), followed by stimulation with TNF at the indicated concentrations. After 24 h, apoptosis was scored by morphological assessment. Data represent triplicate counts of a minimum of 300 cells per treatment. **(B)** Primary HUVEC cells were left untreated, or treated for 2 h with BV6 (4 μM), followed by addition of TNF (10ng/ml). After 24 h, cells were viewed by phase contrast microscopy.

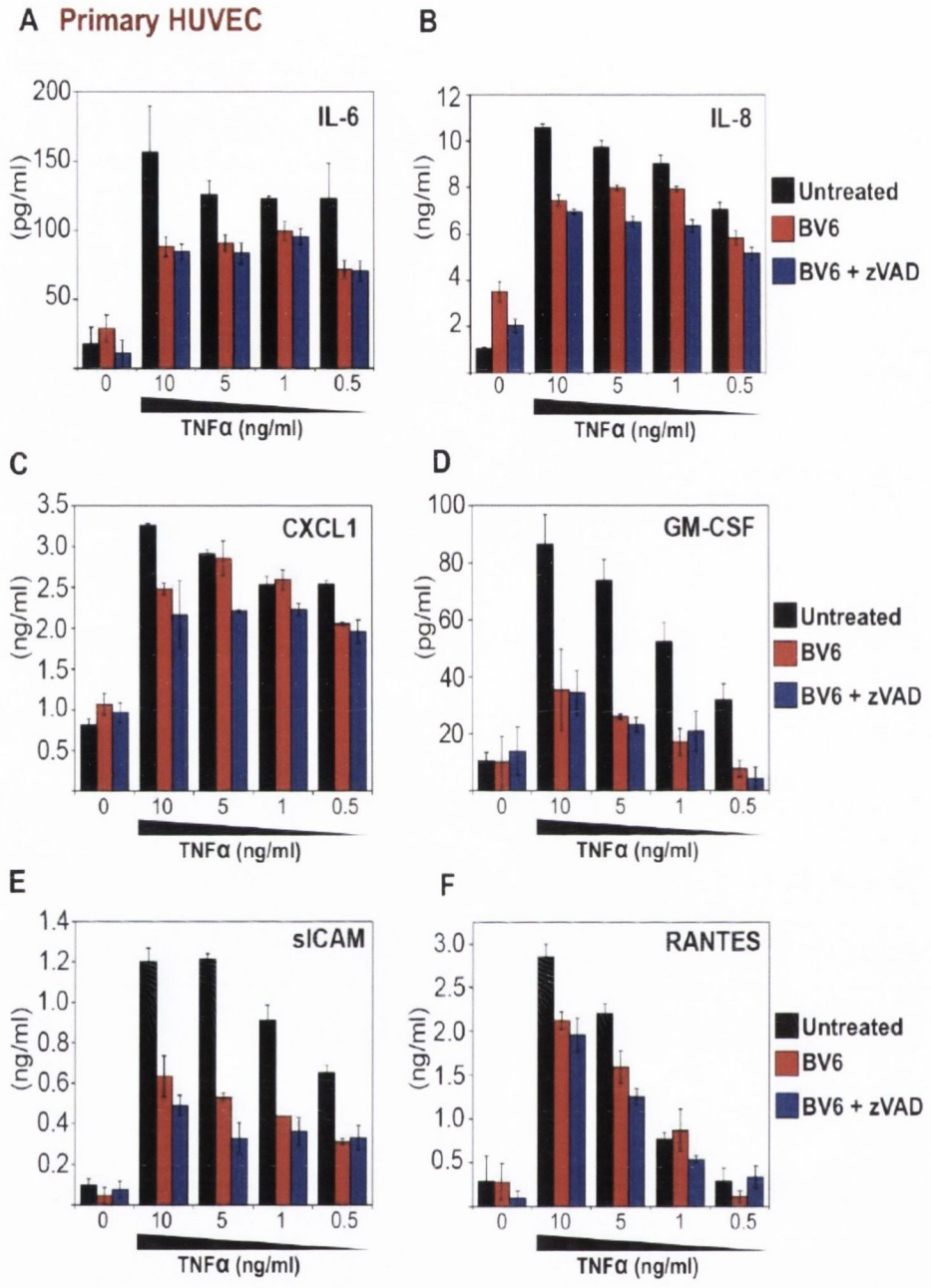

Figure 3.6

IAP antagonist-mediated suppression of inflammatory cytokines can be uncoupled from sensitization towards apoptosis.

(A-F) Primary HUVEC cells were either left untreated or treated for 2 h with BV6 (4 μM) in the presence or absence of zVAD-fmk (20 μM), followed by stimulation with TNF at the indicated concentrations. After 24 h,cytokine concentrations in the culture supernatants from were determined by ELISA. Results shown are representative of least three independent experiments. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

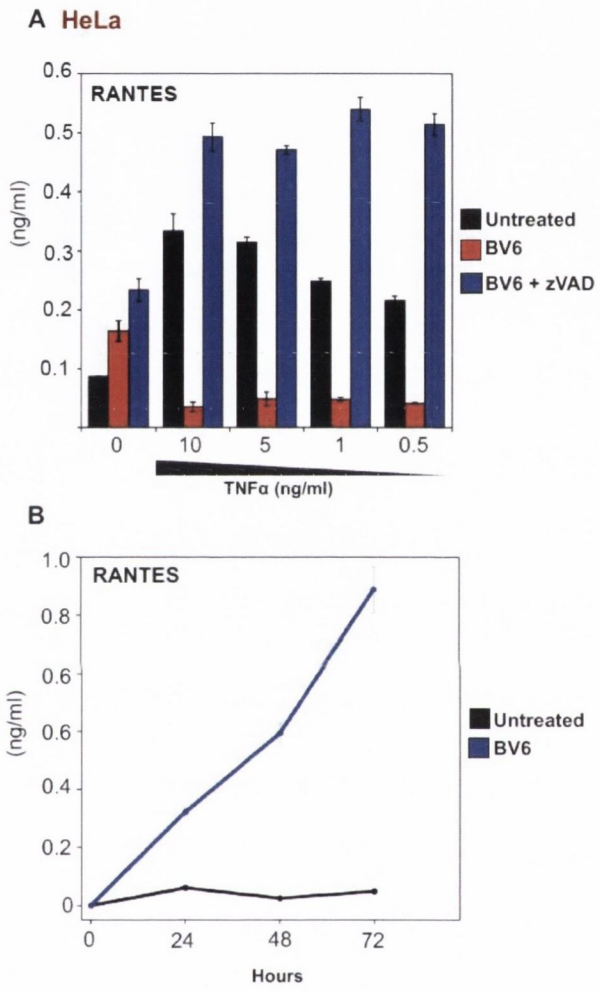

Figure 3.7

IAP depletion leads to spontaneous release of RANTES. (A) HeLa cells were either left untreated or treated with BV6 (4 μM) in the presence or absence of zVAD-fmk (20 μM) for 2 h, followed by stimulation with TNF at the indicated concentrations. After 24 h, RANTES concentrations in the culture supernatants were determined by ELISA. **(B)** HeLa cells were left untreated or treated with BV6 (2 μM). At the indicated timepoints, RANTES concentrations in the culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

cytokines and chemokines in response to BV6 treatment in HeLa, HaCaT, and HT-29 cell lines (Figure 3.8 A-C). These experiments revealed that RANTES, as well as IL-8 to a lesser degree, were consistently induced in the presence of IAP antagonist alone (Figure 3.8 A-C), in contrast to the generally suppressive effects seen in response to TNF-induced production of these chemokines (Figure 3.3). Moreover, this effect was observed in several independent cell lines (Figure 3.8 A-C). IAP antagonist-induced expression of RANTES did not appear to be due to TNF production because this was not attenuated through addition of neutralizing anti-TNF antibodies (Figure 3.8 A-C). Thus, interfering with cellular IAPs can produce complex alterations to the pattern of spontaneous as well as TNF-induced production of cytokines and chemokines.

IAP antagonists have been shown to promote NIK stabilization as a consequence of cIAP-1 and cIAP-2 degradation, both of which appear to contribute to constitutive degradation of this kinase (Varfolomeev et al., 2007). Therefore, we considered the possibility that BV6-induced chemokine production may be due to the activation of NIK-dependent non-canonical NFκB (Dillon et al., 2012). To explore this, we monitored the processing of NFκB2 p100 to p52 during BV6 treatment and confirmed that this indeed occurred in parallel with RANTES production (Figure 3.9 A), suggesting that non-canonical NFκB activation was responsible for chemokine production as a result of BV6 treatment. However, knockdown of p100 failed to suppress BV6-induced RANTES production, whereas knockdown of RelA/p65 sharply attenuated production of this chemokine under the same conditions (Figure 3.9 B). This suggests that BV6 induces a complex route to selective NFκB p65 activation, possibly due to the elimination of p100 which as been reported to act as a *bona fide* inhibitor of a fraction of cytoplasmic RelA/p65 (Basak et al., 2007).

3.2.5 TNF-dependent cytokine production is regulated through RIPK1

cIAP-1 and cIAP-2 have been implicated as regulators of RIPK1 polyubquitination and recruitment of downstream signaling intermediates in the context of TNFR signaling (Bertrand et al., 2008; Vucic et al., 2011). Because the preceding

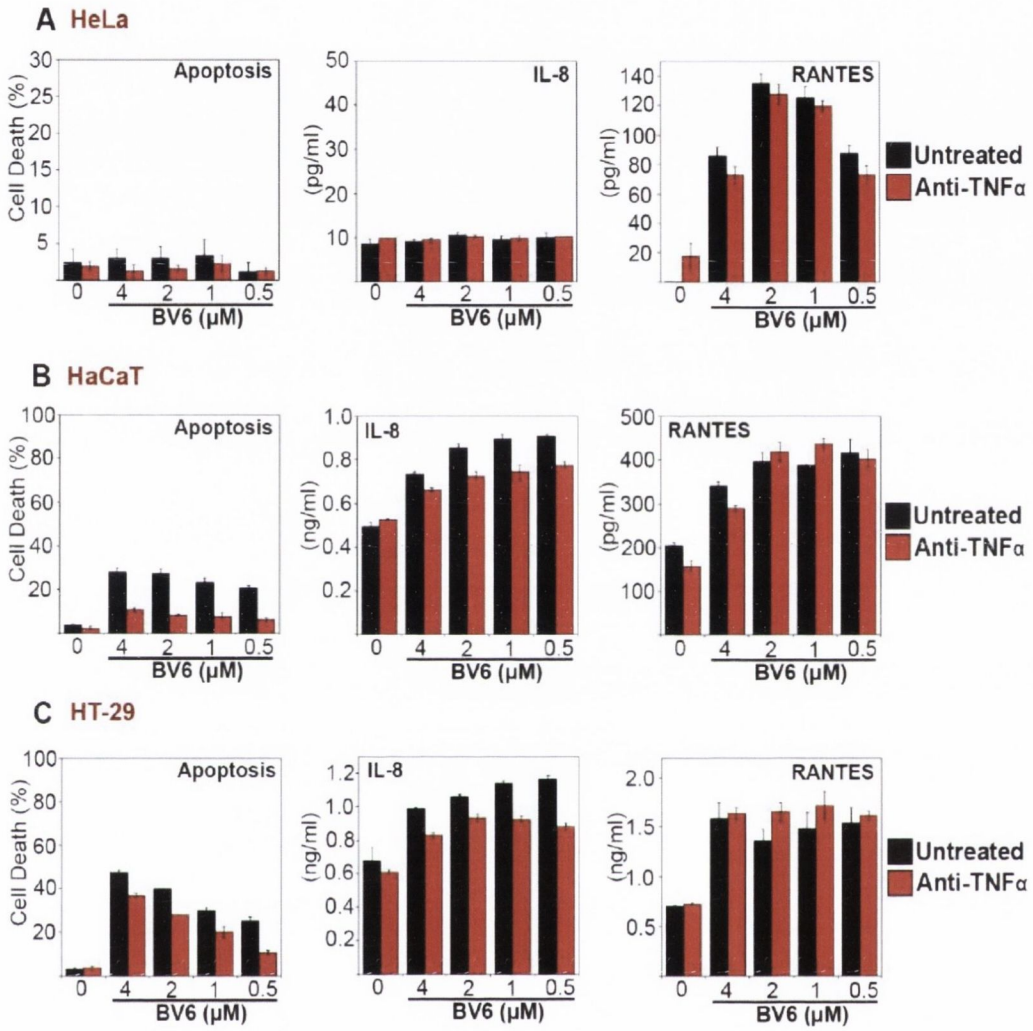

Figure 3.8

IAP antagonism can lead to spontaneous production of chemokines.

(A-C) The indicated cell lines either left untreated or treated with neutralizing anti-TNF antibodies (1 μg/ml) for 1 h, followed by addition of BV6 at the indicated concentrations. 24 h later, apoptosis was determined by morphological assessment and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA. Data represent triplicate counts of a minimum of 300 cells per treatment. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

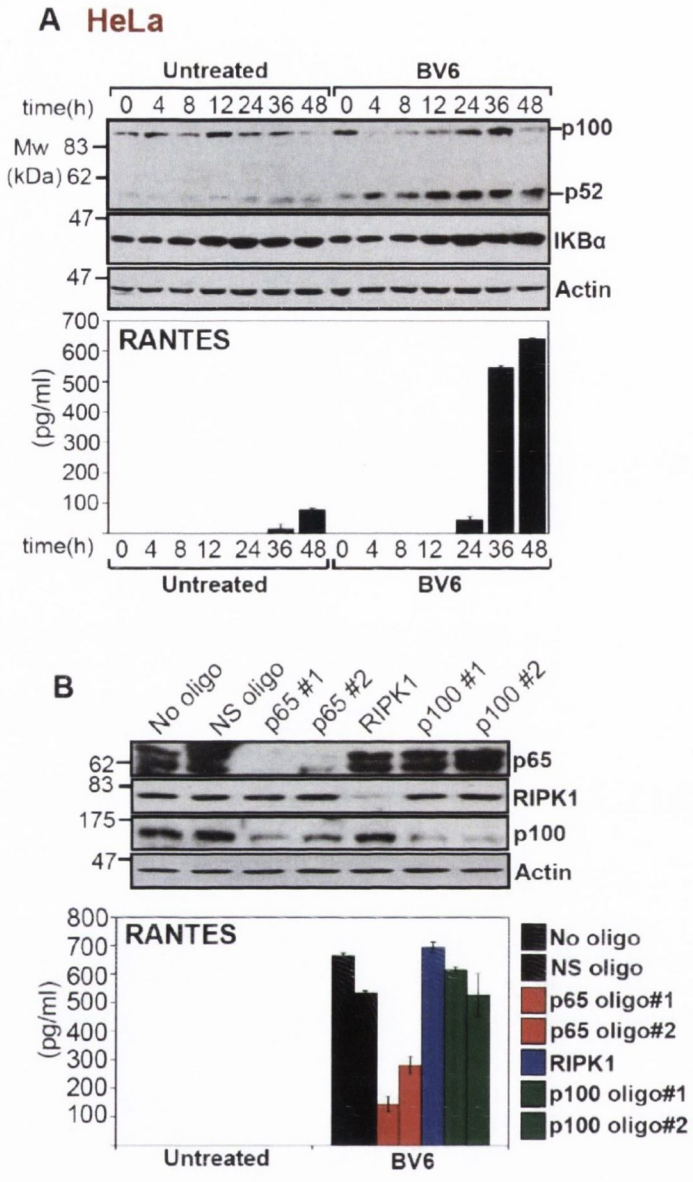

Figure 3.9

IAP antagonism triggers NFκB p65- dependant RANTES production.

(A) HeLa cells were seeded at $1x10^6$ in 6cm plates. The following day, cells were either left untreated or treated with BV6 (2 μM). At the indicated timepoints, cell lysates were analysed by immunoblotting for levels of endogenous p100/p52 and IKBα, while RANTES was measured in the corresponding supernatants by ELISA. **(B)** HeLa cells were transfected either with non-silencing siRNA, or siRNAs targeted against p65, RIPK1 or p100 and 48 h later, cells were either left untreated, or treated with BV6 (2 μM). After a further 48 h, cell lysates were analysed by immunoblotting for the indicated proteins and RANTES concentrations in the supernatants were determined by ELISA. Results shown are representative of at least three independent experiments. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

experiments found that IAP neutralization broadly suppressed TNF-induced cytokine production, this suggested that RIPK1 was important in this context. Thus, we asked whether knockdown of RIPK1 could also inhibit TNF-induced cytokine production. As Figure 3.10 *A* shows, silencing of RIPK1 with two different siRNAs greatly attenuated TNF-induced IL-6, IL-8, and CXCL1 production suggesting that this kinase is required for the pro-inflammatory effects of TNFR stimulation. Consistent with this, transient overexpression of RIPK-1 also promoted production of IL-6, IL-8, CXCL1, MCP-1 and RANTES from HeLa cells (Figure 3.10 B). Moreover, inhibition of RIPK1-induced apoptosis using zVAD-fmk potentiated production of the latter cytokines, as expected (Figure 3.10 B), by restoring cell viability. RIPK1-induced production of IL-8 and CXCL1 was robustly inhibited through addition of BV6 (Figure 3.11 A), although once again we observed that BV6 enhanced RANTES expression.

Because RIPK1 activity is required for TNF-induced cytokine production as illustrated above, we next explored whether RIPK1-induced production of IL-6, IL-8 and CXCL1 could be enhanced through co-expression of cIAP-1, cIAP-2 or XIAP. As Figure 3.11 *A* illustrates, co-transfection of cIAP-1 and cIAP-2 as well as XIAP along with RIPK1 led to enhanced IL-6, IL-8, and CXCL1 production.

3.2.6 Knockdown of cIAP-2 attenuates RIPK1- and TNF-induced cytokine production

We next explored whether all three IAPs were required for optimal RIPK1-dependent production of cytokines through knocking down endogenous cIAP-1, cIAP-2 and XIAP, followed by transfection of RIPK1 (Figure 3.12). As Figure 3.12 *B-D* illustrates, knockdown of cIAP-2 had the greatest effect on RIPK1-induced cytokine production, with knockdown of both cIAP-1 and cIAP-2 having a greater effect than either alone. By contrast, knockdown of XIAP resulted in only a modest decrease in RIPK1-dependent cytokine production (Figure 3.12 B-D). These data suggest that RIPK1-dependent cytokine production requires endogenous cIAP-1 and cIAP-2.

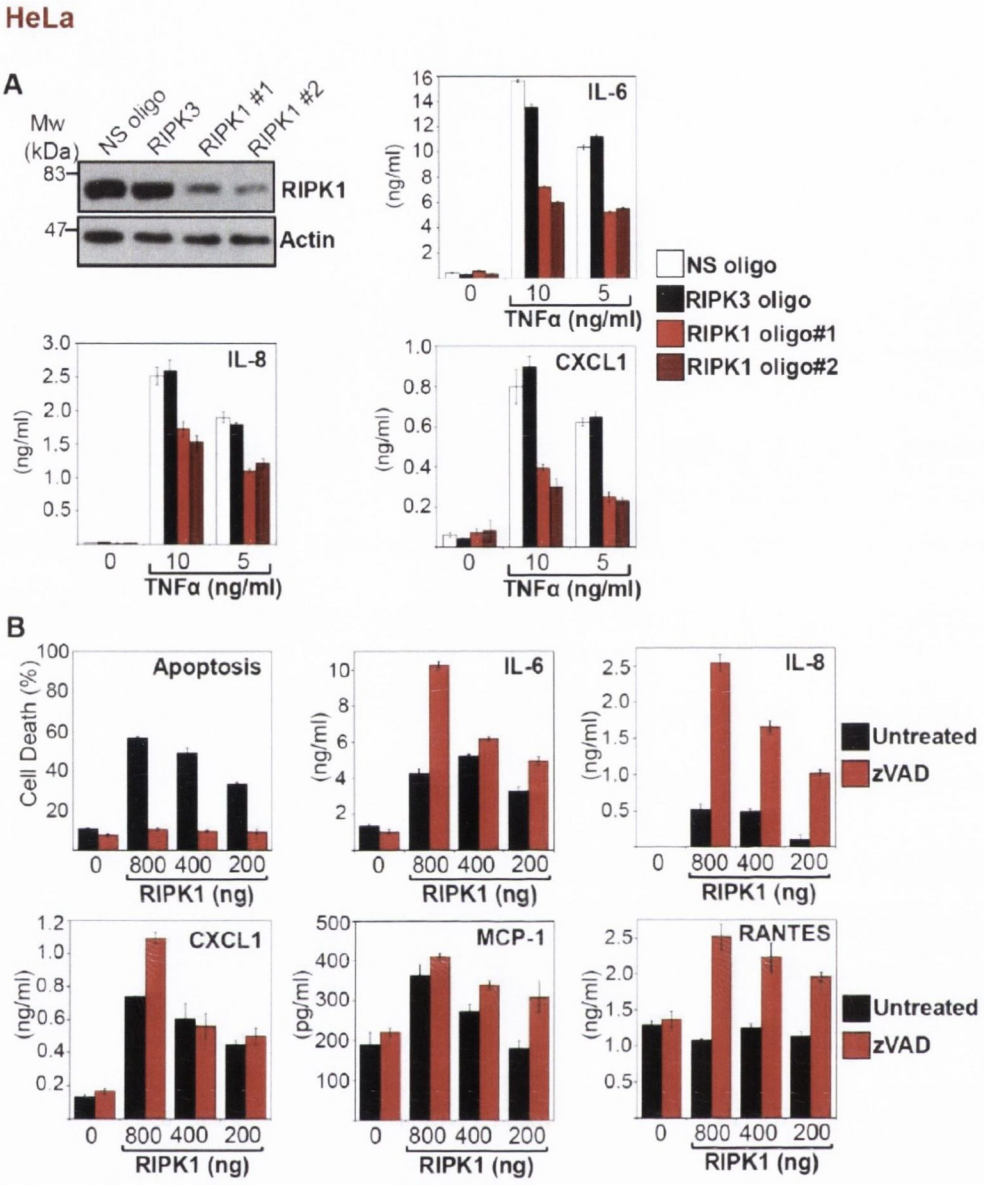

Figure 3.10

RIPK-1 plays a central role in TNF-induced cytokine production. (A) HeLa cells were transfected with either non-silencing, RIPK3 or RIPK1-targeted siRNAs then 48 h later, cells were treated with the indicated concentrations of TNF. After a further 24 h, cell lysates were analysed by immunoblotting for levels of endogenous RIPK1 and cytokine concentrations in the supernatants were determined by ELISA. **(B)** HeLa cells were transfected with empty vector or the indicated concentrations of a cDNA encoding RIPK1 in the presence or absence of zVAD-fmk (20 μM). After 24 h, apoptosis was determined by morphological assessment and cytokine concentrations in the culture supernatants were determined by ELISA. . Data represent triplicate counts of a minimum of 300 cells per treatment. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

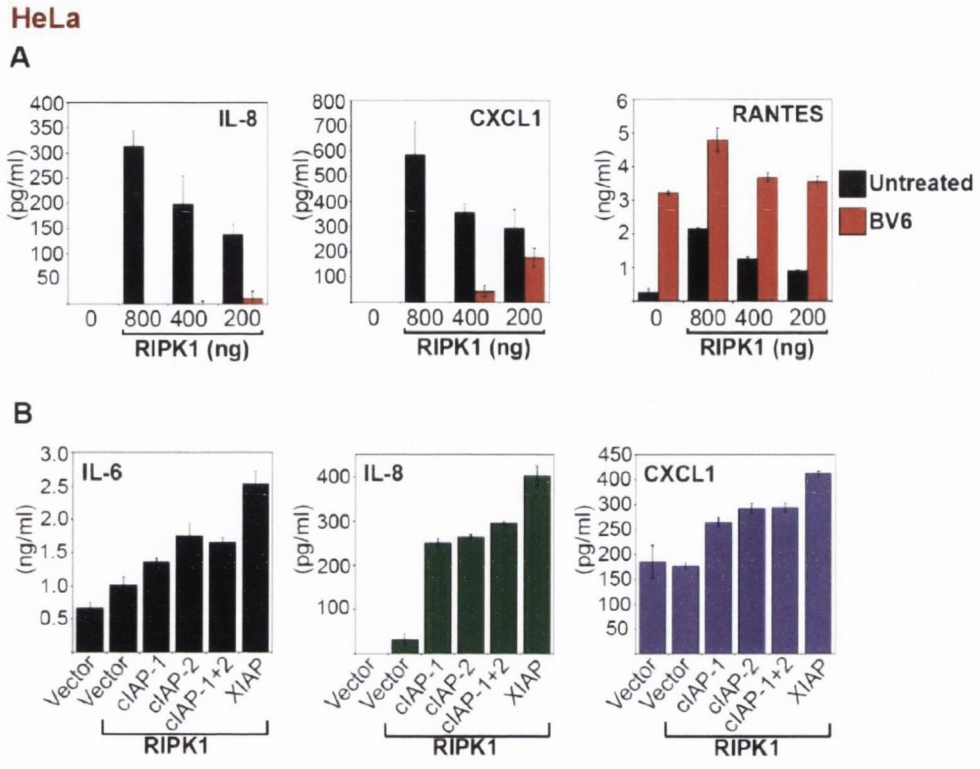

Figure 3.11

IAPs cooperate with RIPK1 to drive optimal cytokine production. (A) HeLa cells were transfected with empty vector or the indicated concentrations of a cDNA encoding RIPK1 in the presence or absence of BV6 (2 μM). zVAD-fmk (20 μM) was used to suppress RIPK1 induced cell death. After 24 h, cytokine concentrations in the culture supernatants were determined by ELISA. **(B)** HeLa cells were pre-treated for 12 h with BV6 (1 μM) then transfected in fresh medium with empty vector, RIPK1 cDNA alone or RIPK1 cDNA in combination with IAP cDNA as indicated. After 24 h, cytokine concentrations in the culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

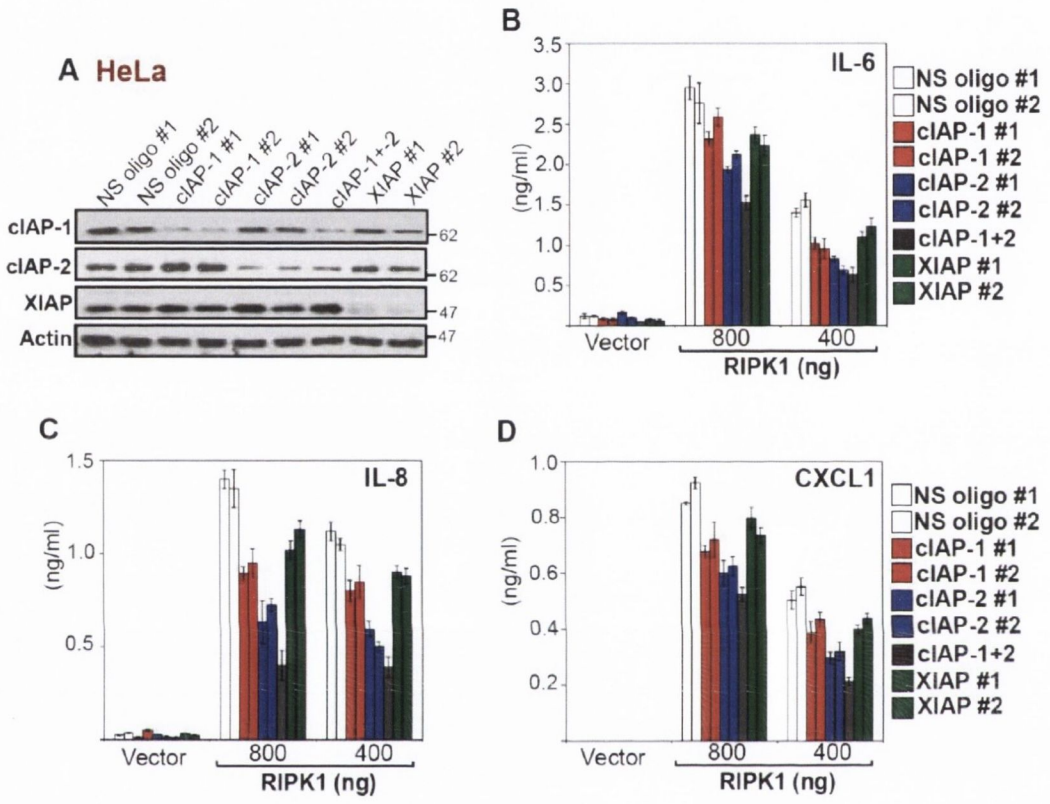

Figure 3.12

Knockdown of endogenous IAPs attenuates RIPK1- induced inflammatory responses.

(A) HeLa cells were electroporated with either non-silencing siRNA or siRNAs directed against cIAP-1, cIAP-2 or XIAP, as indicated. After 48 h, cells were transfected with empty vector or a cDNA encoding RIPK1 in the presence of zVAD-fmk (25 μM), for inhibition of RIPK1-mediated cell death. 24 h later, cell lysates were analysed by immunoblotting for endogenous IAPs and cytokine concentrations in the culture supernatants were determined by ELISA **(B-D).** Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

To confirm the identity of the endogenous IAPs required for optimal TNF-induced cytokine production, we again silenced expression of endogenous XIAP, cIAP-1 and cIAP-2 using specific siRNAs, followed by TNF stimulation. Similar to the effects of using IAP antagonist (BV6), knockdown of cIAP-2 attenuated TNF-induced IL-6, IL-8 and CXCL1 secretion and sensitized towards TNF-induced apoptosis (Figure 3.13). Taken together, these data argue that cIAP-2 in particular is important for optimal TNF-induced cytokine production.

3.2.7 Several TNF-induced kinases are suppressed through IAP antagonism

TNF promotes the production of pro-inflammatory cytokines and chemokines through activating several kinases, including IKK kinases, p38MAPK, MEK/ERK and JNK. Because TNF-induced cytokine production was attenuated through IAP antagonism, this suggested that one or more TNF-induced kinases failed to be activated upon loss of IAPs. To explore this issue, we monitored TNF-induced signal transduction events in the presence and absence of IAP antagonist. As Figure 3.14 illustrates, TNF-induced activation of NFκB, MEK/ERK, p38MAPK and JNK were all greatly attenuated in the presence of BV6. Furthermore, using a panel of kinase inhibitors (Figure 3.15), similar inhibition of TNF-induced IL-6 and IL-8 production was also observed due to inhibition of p38 MAPK, and of MEK/ERK to a lesser degree (Figure 3.15). Thus, neutralization of IAPs suppresses TNF-induced production of cytokine and chemokines, most likely as a consequence of interfering with the activation of multiple kinases downstream of TNF receptor engagement.

3.2.8 IAPs facilitate TNF-induced MCP-1 production, which is required for phagocyte chemotaxis

As we had observed a dramatic attenuation of TNF-induced chemokines in the presence of BV6, we explored if IAP antagonism could attenuate TNF-induced chemotaxis of phagocytes. To this end, we utilized an *in vitro* chemotaxis assay in which the ability of THP-1 monocytes to migrate towards cell supernatants was monitored. Indeed, supernatants derived from HeLa cells treated with TNF promoted robust chemotaxis of THP-1 cells, compared to untreated HeLa

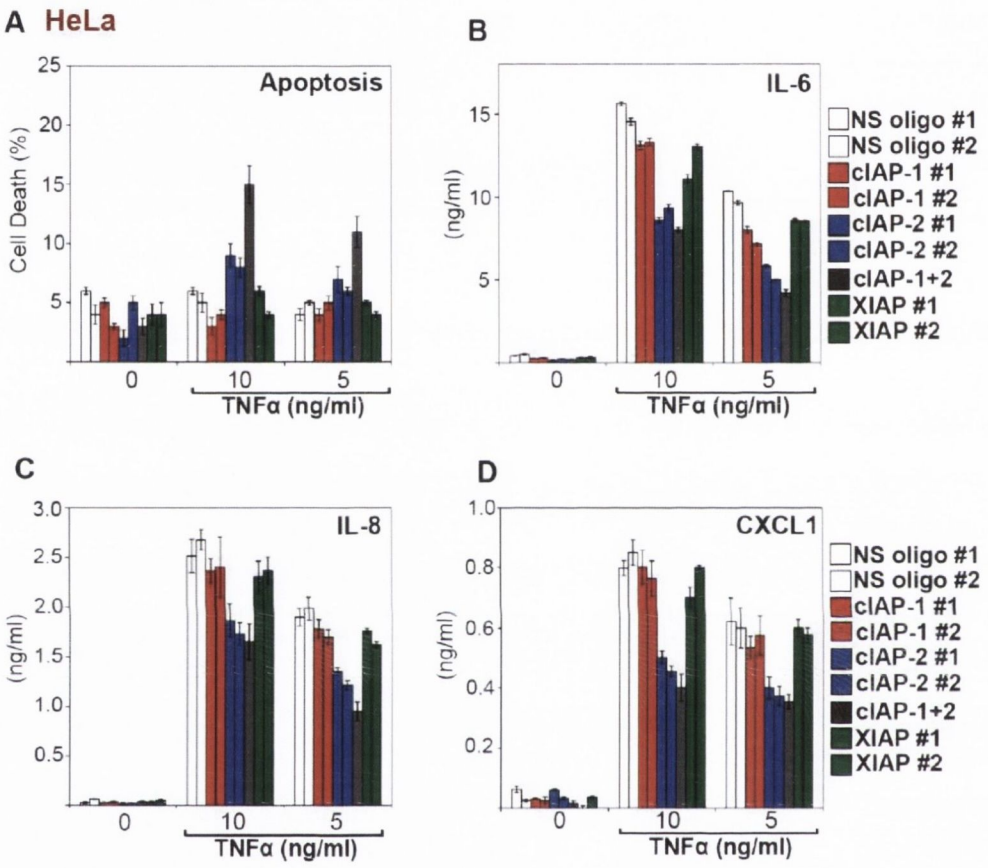

Figure 3.13

Knockdown of endogenous IAPs attenuates TNF- induced inflammatory responses. (A) HeLa cells were electroporated with either non-silencing siRNA, or siRNAs directed against cIAP-1, cIAP-2 or XIAP, as indicated. 48 h later, cells were treated with the indicated concentrations of TNF. After 24 h, cell death was scored by annexin V/PI staining and cytokine concentrations in the resulting supernatants were determined by ELISA **(B-D)**. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

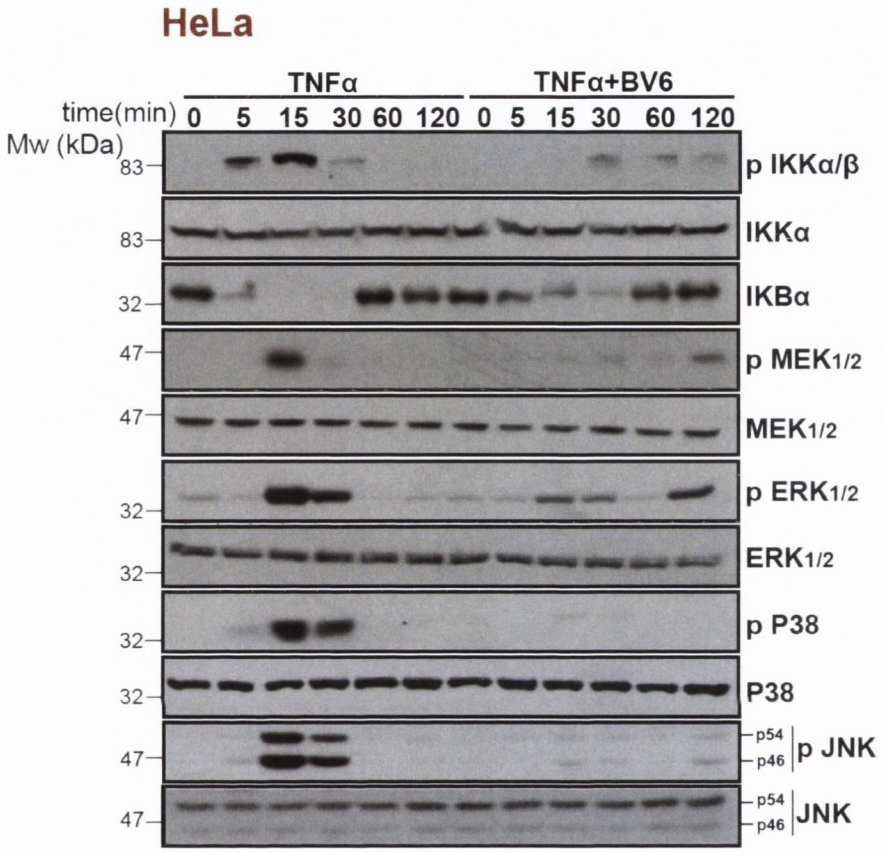

Figure 3.14

TNF-induced activation of multiple kinases is inhibited through neutralization of IAPs. HeLa cells were either left untreated, or treated with BV6 (2 μM) for 1 h, followed by addition of TNF (10 ng/ml). Cell lysates were prepared at the indicated time points and analysed by immunoblotting for the indicated proteins.

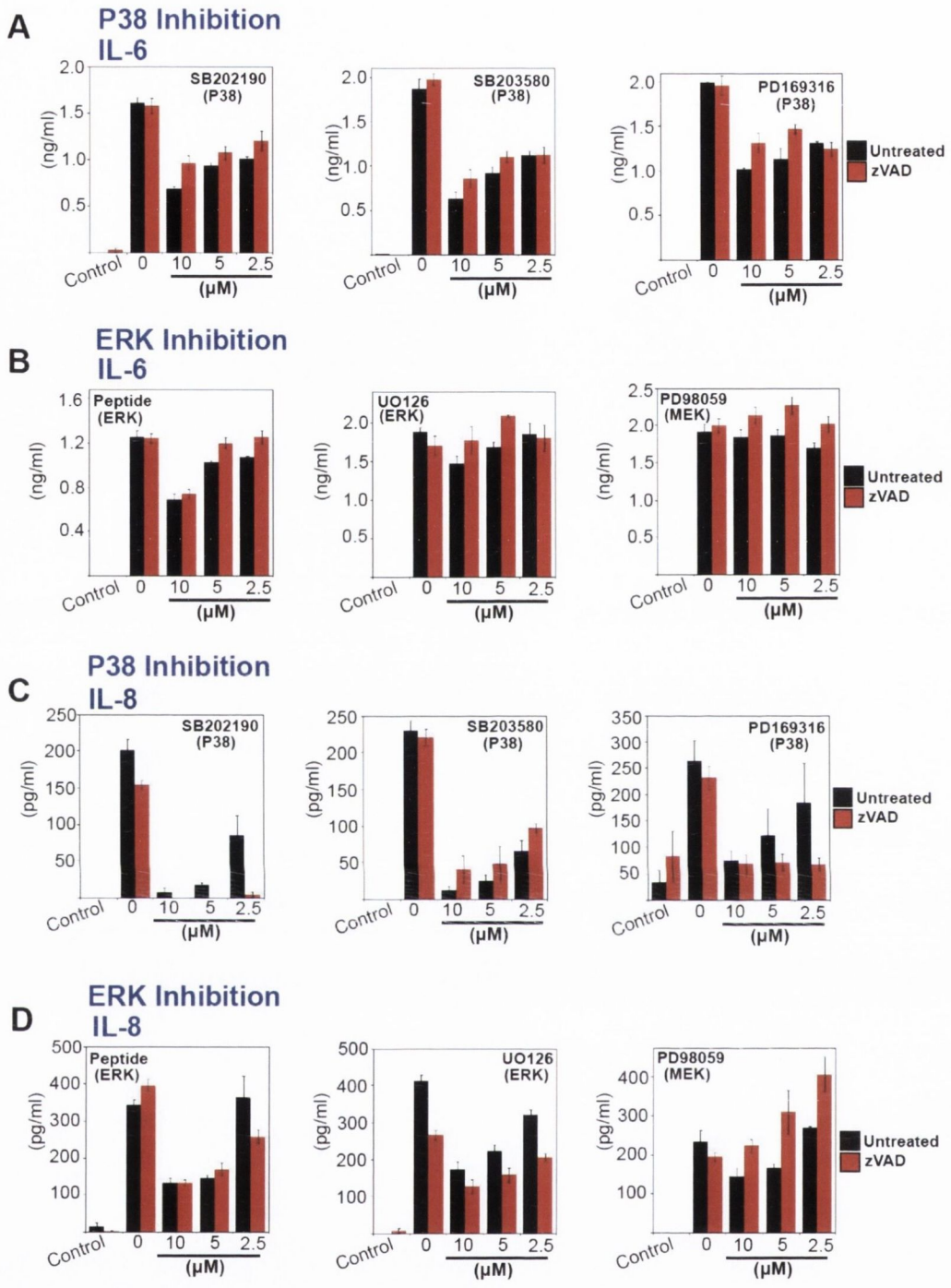

Figure 3.15

TNF-induced IL-6 and IL-8 secretion is attenuated through P38 MAPK inhibition. (A, B) HeLa cells were either left untreated, or treated for 2 h with the indicated concentration of kinase inhibitor in the presence or absence of zVAD-fmk (10 μM), followed by addition of TNF (1 ng/ml). After 24 h, IL-6 concentrations in the culture supernatants were determined by ELISA. **(C, D)** HeLa cells were treated as in *A* and *B*, followed by measurement of IL-8 in the culture supernatants. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

supernatants (Figure 3.16 A). As expected, addition of BV6 attenuated the chemotactic potential of TNF treated HeLa cells, indicating an IAP dependent factor(s) was promoting THP-1 recruitment (Figure 3.16 B). To identify such a factor, we depleted TNF treated HeLa supernatants of likely candidates, finding that MCP-1 was largely responsible for promoting THP-1 chemotaxis (Figure 3.16 C). This was consistent with the dramatic suppression of MCP-1 secretion observed in the presence of BV6 (Figure 3.3). Furthermore, knockdown of RIPK1 and p38 MAPK attenuated the release of chemotactic factors from HeLa cells (Figure 3.16 D).

3.2.9 IAP antagonism can influence TNF-induced inflammatory responses *in vivo*

We next explored whether IAP antagonism could influence the pro-inflammatory effects of TNF *in vivo* by administering recombinant TNF into the peritoneal cavity of wild type mice in the presence and absence of BV6. As expected, TNF-treatment led to a rapid influx of neutrophils into the peritoneum (Figure 3.17 and 3.18). We confirmed these results using cytospins, which also revealed an increase in the percentage of peritoneal lymphocytes (Figure 3.19). As expected, TNF administration induced elevated levels of IL-6, MCP-1, M-CSF, G-CSF, RANTES and IL-1α in the peritoneal lavage fluid (Figure 3.20). However, TNF-induced recruitment of neutrophils was slightly enhanced in the presence of BV6, and treatment with this IAP antagonist alone also elevated neutrophil counts in the peritoneum (Figure 3.17, 3.18 and 3.19). The latter observation correlated with elevated production of the granulocytic chemokine, RANTES, in the peritoneal lavage fluid (Figure 3.20), consistent with what we had earlier observed *in vitro*. Furthermore, co-administration of BV6 with TNF robustly inhibited TNF-induced IL-6 production (Figure 3.20), with more modest inhibitory effects observed with MCP-1 and M-CSF, while production of other cytokines and chemokines was either unaffected or slightly enhanced due to BV6 co-administration (Figure 3.20). Once again, these data demonstrate that interfering with IAP function can influence spontaneous as well as TNF-driven inflammatory responses.

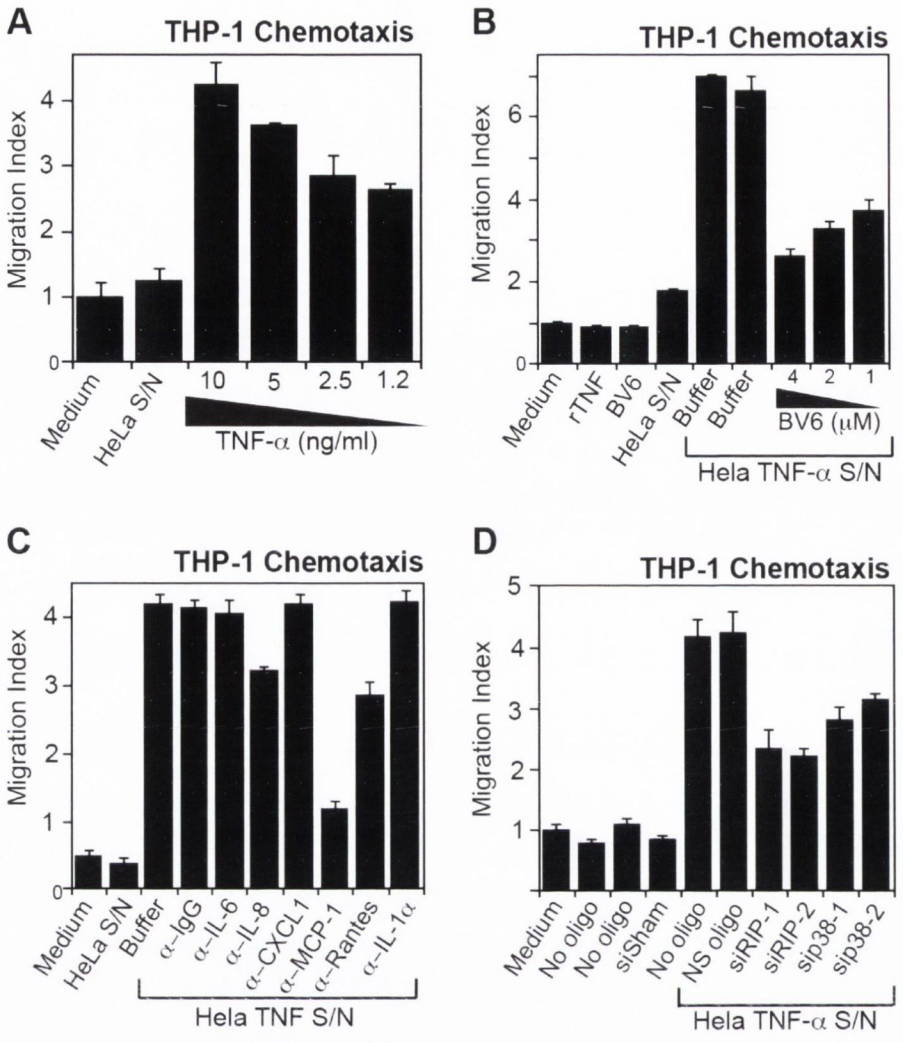

Figure 3.16

TNF induces cIAP and RIPK1-dependant release of MCP-1, that promotes phagocyte recruitment. (A) HeLa cells were either unstimulated (HeLa S/N) or were treated with TNF at the indicated concentrations, in RPMI, supplemented with 0.5% FCS. Twenty-four hours later, THP-1 cells were used for the chemotaxis assay, separated from the cell supernatants by 8 mM nitrocellulose filters. Cells that had migrated into the bottom wells of the chemotaxis chambers were counted after 1–2 hr and are expressed relative to the migration observed in the control wells. **(B)** HeLa cells were either left untreated (HeLa S/N) or treated with BV6 at the indicated concentrations for 1 h, followed by addition of TNF (10 ng/ml). After 24 h, cell supernatants were used for a chemotaxis assay, as in *A*. BV6 (4uM) and TNF (10 ng/ml) in assay buffer were used as controls. **(C)** HeLa cells were either unstimulated (HeLa S/N), or were treated with TNF (10 ng/ml). Resulting supernatents were immune-depleted of the indicated cytokines using specific antibodies coupled to A/G agarose beads. Supernatents were then used for the chemotaxis assay, as described in *A*. **(D)** HeLa cells were electroporated with mock, non-targeting siRNA (sham), or siRNA directed against RIPK1 and P38 MAPK. After 48 h, cells were either left untreated, or treated with TNF (10 ng/ml) for 24 h. Resulting supernatants were then used in the chemotaxis assay, as described in *A*.

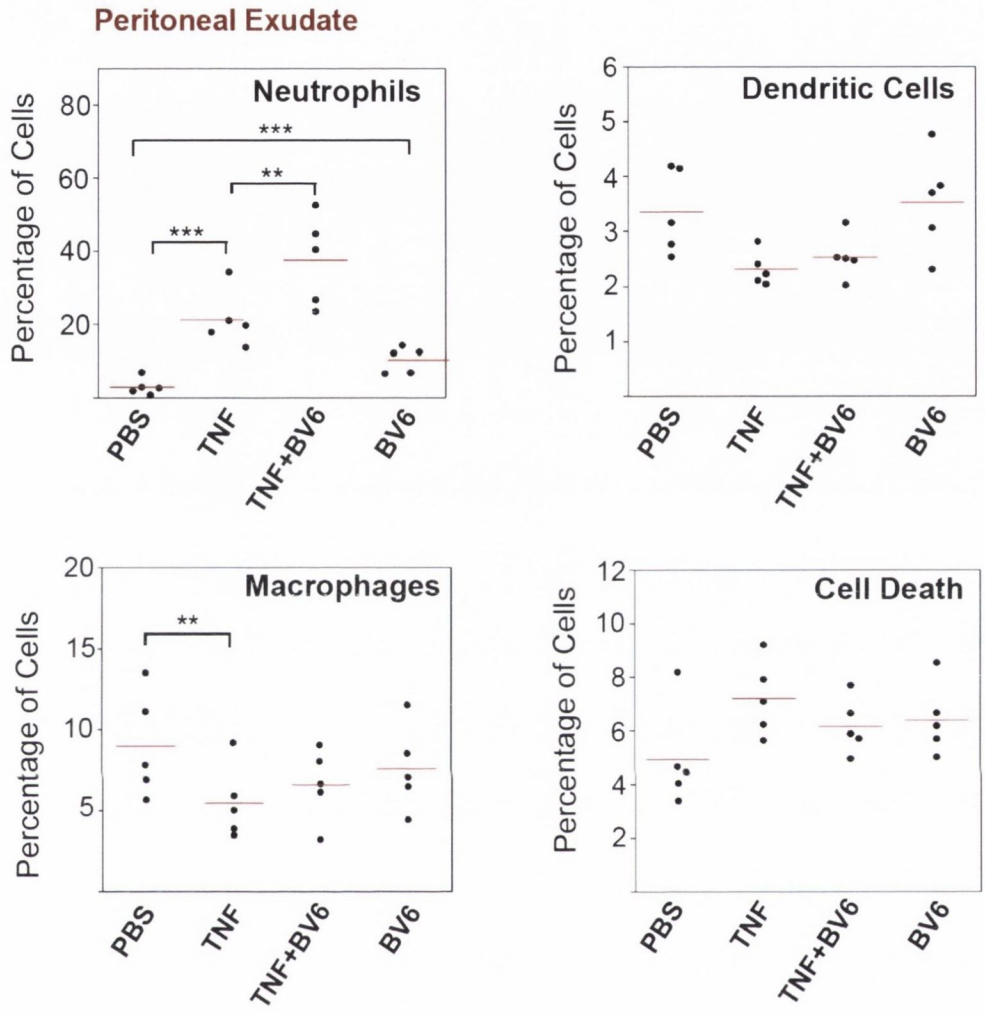

Figure 3.17

IAP antagonism modulates TNF-induced recruitment of immune cells to the peritoneum. (A) Female C57BL/6 mice (5 per treatment group) were injected (i.p. 200 μl) with PBS or BV6 (20 μM). After 2 h, mice were injected again with either PBS or TNF (1 μg per mouse). After a further 10 h, mice were sacrificed and the peritoneal cavity was washed with 5 ml of PBS. Peritoneal lavage-derived cells were immunostained and analysed by flow cytometry for changes in population percentages. Percentage cell death was determined by Aqua positivity. Error bars represent the mean ±SEM. *p<0.1; **p<0.05; ***p<0.01 by Student's t test.

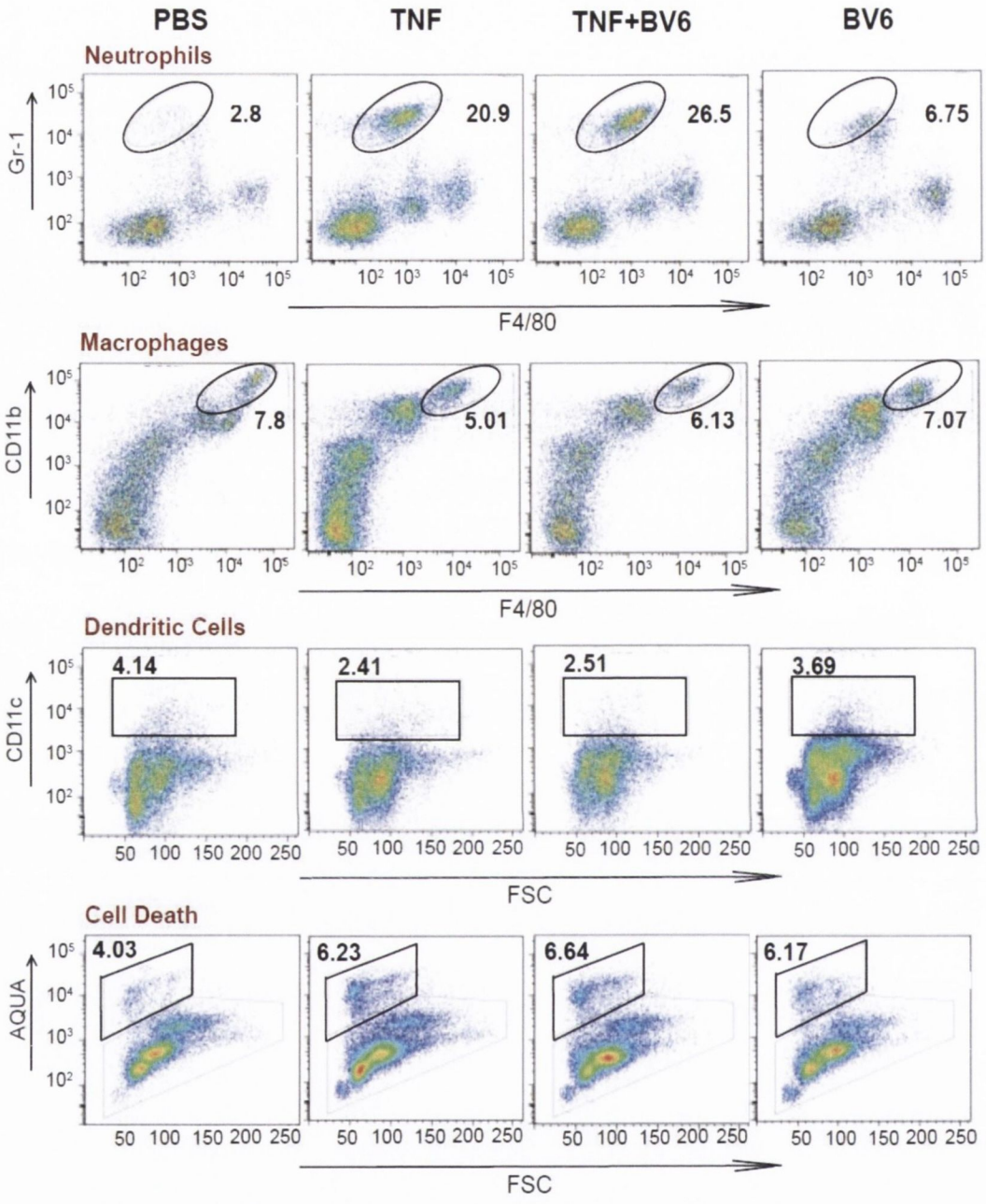

Figure 3.18

Flow cytometry analysis of peritoneal exudates from mice treated with TNF +/- BV6. Representative flow cytometry charts from experiment described in Figure 3.16. Peritoneal lavage derived neutrophils were gated as CD11b+, Gr1 high, F4/80+. Peritoneal lavage derived macrophages were gated as CD11b high, F4/80 high. Peritoneal lavage derived dendritic cells were gated as CD11c high. Dead peritoneal cells were gated as AQUA+, forward scatter low.

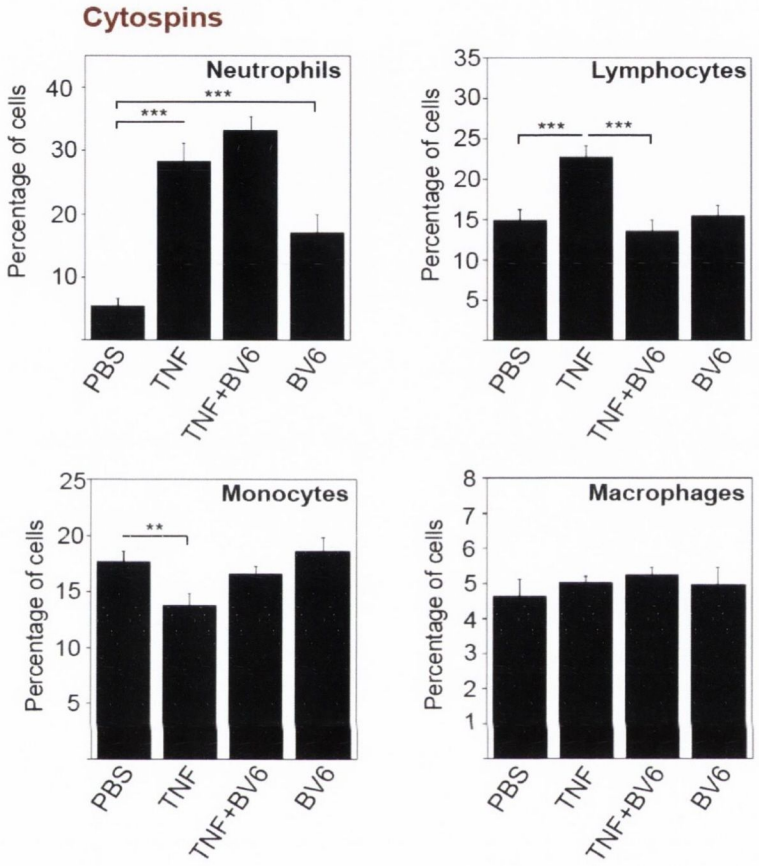

Figure 3.19

Cytospin analysis of peritoneal exudates from mice treated with TNF +/- BV6. Female C57BL/6 mice (5 per treatment group) were injected (i.p. 200 μl) with PBS or BV6 (20 μM). After 2 h, mice were injected again with either PBS or TNF (1 μg per mouse). After a further 10 h, mice were sacrificed and the peritoneal cavity was washed with 5 ml of PBS. Peritoneal exudates were adjusted to $1x10^6$ ml and 200 ul used to make cytospins. Cytospins were stained with haemotoxylin and eosin and used to identify immune cell types by typical morphology. A minimum of 100 cells was counted per slide, in triplicate. Error bars represent the mean ±SEM. *p<0.1; **p<0.05; ***p<0.01 by Student's t test.

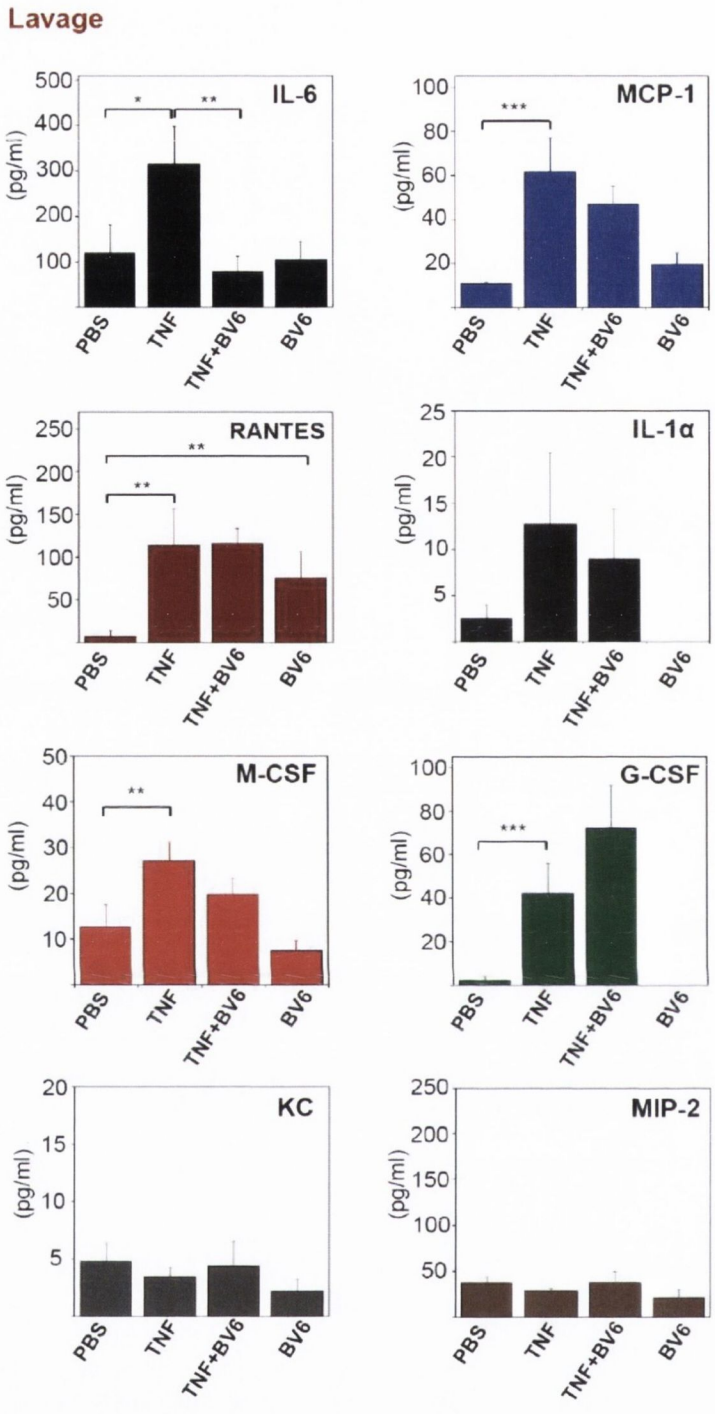

Figure 3.20

IAP antagonism modulates TNF-induced cytokines and chemokines in vivo. Female C57BL/6 mice (5 per treatment group) were injected (i.p. 200 μl) with PBS or BV6 (20 μM). After 2 h, mice were injected again with either PBS or TNF (1 μg per mouse). After a further 10 h, mice were sacrificed and the peritoneal cavity was washed with 5 ml of PBS. Cytokine/chemokine concentrations in the peritoneal lavage fluid were determined by ELISA. Error bars represent the mean ±SEM. *p<0.1; **p<0.05; ***p<0.01 by Student's t test.

Collectively, these data indicate that, in addition to their role as arbiters of cell death, IAPs also contribute to the regulation of spontaneous as well as TNF-induced cytokine and chemokine production, a property that may have significant implications for use of IAP antagonists as therapeutic agents.

3.3 DISCUSSION

Here we have shown that interfering with IAP function, either through knockdown of IAPs, or use of a small molecule IAP antagonist, can influence TNF-induced production of a range of pro-inflammatory cytokines and chemokines such as IL-6, IL-8, GM-CSF, CXCL1 and RANTES. These effects were independent of the ability of IAP antagonists to sensitize target cells towards apoptosis. However, IAP antagonism also led to spontaneous production of chemokines, such as RANTES, both *in vitro* as well as *in vivo*. Because BV6 induced degradation of cIAP1, cIAP2, and XIAP, we used siRNA-mediated knockdown to identify cIAP2 as the critical regulator of TNF-induced cytokine production. Consistent with the model that cIAPs promote RIPK1 ubiquitination to activate NFκB transcription factors, knockdown of RIPK1 blunted cytokine production, while overexpression of RIPK1 drove cytokine production. Furthermore, overexpression of IAPs, enhanced RIPK1-dependant cytokine production. Taken together, we conclude that cIAPs are critical regulators of TNF-induced cytokine production, in a RIPK1 dependent manner.

3.3.1 IAP antagonists as anti-inflammatory agents

A number of chronic inflammatory conditions, such as rheumatoid arthritis, Crohn's disease and psoriasis, are associated with overproduction of TNF, resulting in accumulation of downstream cytokines and chemokines and migration of innate immune cells into the affected area (Brennan and McInnes, 2008). Consequently, biologics such as anti-TNF antibodies capable of inhibiting the actions of this cytokine exhibit considerable therapeutic benefit (Scheinecker et al., 2008). TNF exerts its pathologic effects in such conditions through a cascade effect, which elicits the production of additional cytokines and chemokines from a variety of cell types, thereby provoking further escalation of immune responses. Our observations

that small molecule IAP antagonists can attenuate some of the pro-inflammatory effects of TNF suggest that targeting IAPs may be a useful strategy in inflammatory diseases. Indeed, our data support findings by Mayer et al., 2013, in which administration of an IAP antagonist to mice attenuated antigen-driven arthritis and leukocyte migration into TNF-activated muscle.

Importantly, IAPs have also been shown to be required for optimal inflammasome activation and mature IL1β release (Labbe et al., 2011). As TNF and IL1β are perhaps the most inflammatory apical cytokines, agents that that attenuate their effects are likely to act as potent inhibitors of innate immune reactions. While we have demonstrated that cIAPs are critically involved in TNF-induced cytokine and chemokine production, XIAP is known to be required for NOD receptor-induced NFκB activation and cytokine secretion following bacterial detection (Damgaard et al., 2012). Therefore, IAP antagonists that target XIAP for proteasomal degradation, such as BV6 may be useful therapeutically to dampen sepsis as a result of excessive NOD receptor stimulation.

3.3.2 Complications for the use of IAP antagonists in cancer treatment

IAP antagonists represent a potential new class of anti-cancer drugs, due to their ability to selectively induce TNF-dependent apoptosis of cancer cells (LaCasse et al., 2008). However, our observation that IAP antagonism can also lead to spontaneous production of chemokines, such as RANTES and IL-8, from certain cell types, both *in vitro* as well as *in vivo*, is a potentially significant complicating factor for the use of such agents. Indeed, RANTES production may be a beneficial side effect of IAP antagonists, by facilitating T-cell and NK cell infiltration into the tumor microenvironment to aid in tumor clearance.

IAP antagonists are also under investigation for their ability to provoke apoptosis of tumors in combination with other cytotoxic drugs. Where IAP antagonists display single agent efficacy, this has been shown to be due to sensitization of such tumors to a TNF-dependent autocrine loop where cells already producing TNF now become sensitized to this cytokine due to elimination of the IAP-mediated survival

pathway. TNF has also been implicated in promoting tumor initiation and progression via a process dubbed 'smouldering inflammation' which can recruit cells of the innate immune system to the tumor site as a consequence of production of cytokines and chemokines such as IL-6 and IL-8 (Mantovani et al., 2008). Innate immune cells such as neutrophils and macrophages are capable of provoking further mutations as a consequence of the production of reactive oxygen, and can affect tumor progression through release of additional growth promoting cytokines and chemokines such as IL-6, IL-8 and CXCL1/KC that can have direct effects on tumor cell proliferation, resistance to apoptosis, and can instigate a wound healing response that can promote local neovascularization. Thus, the use of agents that can suppress the pro-inflammatory effects of TNF, in addition to sensitizing tumor cells towards apoptosis, can simultaneously achieve two desirable goals at once; lowering the threshold for apoptosis and breaking the inflammatory cycle that can permit tumor progression and metastasis.

3.3.3 Signal transduction pathways are activated as a result of IAP loss

Chemokine production such as RANTES and IL-8 as a result of IAP antagonism may be due to the activation of a fraction of RelA/p65 as a consequence of NIK-dependent p100 processing to p52 (Figure 3.9). IAP antagonists have been shown to promote NIK stabilization as a consequence of cIAP-1 and cIAP-2 degradation, both of which appear to contribute to constitutive degradation of this kinase (Varfolomeev et al., 2007; Zarnegar et al., 2008). However we found that p100 was not required for BV6-induced RANTES production, apparently ruling out a role for non-canonical NFκB here. Surprisingly, RelA/p65 was involved in spontaneous RANTES production upon treatment with BV6 alone (Figure 3.9). This suggests that IAP antagonists can induce a complex route to RelA/p65 activation, possibly due to the elimination of p100 which has been reported to act as a *bona fide* inhibitor of a fraction of cytoplasmic RelA/p65 (Basak et al., 2007). We have found that BV6-induced RANTES production is RIPK1-independent (Figure 3.9). As TNF-induced chemokine production was RIPK1-dependant, this rules out an autocrine TNF loop in driving BV6-induced RANTES production. We also confirmed this result using anti-TNF neutralizing antibodies.

RIPK1 is likely to be the key target of IAP ubiquitin ligase activity in the TNF pathway to cytokine production. Indeed, we have also shown here that knockdown of RIPK1 greatly attenuated TNF-induced cytokine production. Moreover, overexpression of RIPK1 was sufficient to promote the production of several pro-inflammatory chemokines (Figure 3.10). In this regard, it is interesting to note that the RIPK1-related kinase, RIPK3, has been identified as a key driver of necroptotic cell death (He et al., 2009; Zhang et al., 2009). Indeed, depletion of IAPs with an IAP antagonist is often required to achieve sufficient RIPK3 activity to drive necroptosis. Thus, it appears that IAPs restrain activation of RIPK3. In this regard, it is possible that deregulated RIPK3 activation may also lead to the transcriptional induction of conventional cytokines and chemokines, as we have shown here for RIPK1. Interestingly, myeloid lineage-specific deletion of XIAP and cIAPs, results in spontaneous TNF-secretion that is attenuated by combined genetic loss of RIPK3. This suggests IAPs restrain RIPK3 from participating in cytokine production (Wong et al., 2014). Therefore, RIPK3 may drive a non-canonical route to p65 activation upon IAP loss, which may explain our findings. The contribution of RIPK3 to inflammatory cytokine production requires further clarification.

In summary, independent of their role as inhibitors of apoptosis, our findings suggest that IAPs are important regulators of TNF-induced inflammatory cytokine and chemokine production. Thus, IAPs serve as key arbiters of inflammation as well as apoptosis.

CHAPTER 4

NECROPTOSIS ABROGATES INFLAMMATION BY TERMINATING TNF- OR LPS-INDUCED CYTOKINE AND CHEMOKINE PRODUCTION

4.1 INTRODUCTION

TNF receptor (TNFR) stimulation can result in apoptosis via TRADD/FADD-dependent recruitment of caspase-8 to the TNFR intracellular death domain (Ashkenazi and Dixit, 1998; Wang et al., 2008), as discussed previously (Figure 1.5). Recent studies have also shown that TNFR engagement can result in a form of programmed necrosis, called necroptosis, where TNF-induced caspase-8 activation is frustrated either using pharmacological or viral-derived caspase inhibitors (He et al., 2009; Lin et al., 2004; Mocarski et al., 2012), or as a consequence of targeted disruption of the *Caspase-8* or *FADD* loci (Dillon et al., 2012; Kaiser et al., 2011; Oberst et al., 2011) (Figure 1.9). Necroptosis is generally viewed as a pro-inflammatory mode of cell death and many studies have concluded that this represents a host response to viral infection that limits viral replication (Cho et al., 2009; Di Paolo et al., 2013; Upton et al., 2010, 2012). However, it is frequently overlooked that the majority of TNF-responsive cell types do not undergo apoptosis, or indeed necrosis, in response to TNFR engagement. Instead, most cells initiate highly robust NFκB-dependent pro-inflammatory responses upon stimulation with this cytokine (Figure 1.3).

TNF stimulation rapidly results in recruitment of 'Complex I' components to the TNF receptor, including TRADD, RIPK1 and TRAF2, which rapidly signal to NFκB activation (Micheau and Tschopp, 2003) (Figure 1.4). Complex I-mediated NFκB activation restrains activation of caspase-8 in a cytosolic 'complex II', comprised of FADD, FLIP, RIPK1 and caspase-8 (Micheau and Tschopp, 2003) (Figure 1.5). To undergo apoptosis in response to TNF, cells typically require sensitization through the use of transcriptional/translational inhibitors or IAP-antagonists that block NFκB activation signals (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Vince et al., 2007). Thus, the primary consequence of TNFR stimulation in responsive cell types is the rapid synthesis and secretion of a diverse array of cytokines and chemokines that collectively trigger the inflammatory response (Esche et al., 2005; Rollins, 1997; Rot and von Andrian, 2004). From this perspective, necroptosis could be interpreted as a strategy by viruses encoding caspase inhibitors to shut down the host pro-inflammatory program by terminating synthesis of pro-inflammatory

cytokines and chemokines.

Key biological outcomes of TNFR stimulation includes the recruitment of peripheral blood neutrophils through the generation of gradients of chemokines such as IL-8/MIP-2, recruitment of monocytes/macrophages (via MCP-1), activation of local macrophages and dendritic cells (via MCP-1 and GM-CSF), induction of monocyte/macrophage differentiation (GM-CSF), activation of local endothelium (Burgess et al., 1980; Esche et al., 2005; Janes et al., 2006), as well as a range of other responses (Brennan and McInnes, 2008; Esche et al., 2005). Therefore, the central role of TNF as a trigger for pro-inflammatory cytokine/chemokine production, rather than apoptosis, is a key issue when evaluating the outcome of interventions, such as caspase inhibition, that impact on TNF-induced signal transduction pathways.

Because necrosis is generally pro-inflammatory, in comparison with apoptosis, it is widely presumed that the switching of TNF-induced signaling to necroptosis exacerbates inflammation (Kaczmarek et al., 2013). The latter view is predicated upon the idea that necrosis results in the release of intracellular constituents, called damage-associated molecular patterns (DAMPs), that promote inflammation (Chen and Nunez, 2010; Matzinger, 1994; Moriguchi et al., 1997). However, the presumption that TNF-induced necroptosis represents a more inflammatory outcome than TNF stimulation alone, fails to take account of the fact that TNF is intrinsically highly pro-inflammatory, as noted earlier. An alternative possibility is that necroptosis may suppress inflammation through abruptly terminating TNF-induced cytokine production. It is not known whether the release of intracellular DAMPs, due to necroptosis-associated cell rupture, compensates for inhibition of the production TNF-induced pro-inflammatory factors. Therefore, it is unclear whether necroptosis suppresses or enhances TNF-induced inflammatory responses (Figure 4.1)

Here we examine the inflammatory outcome of TNF- and LPS-induced necroptosis

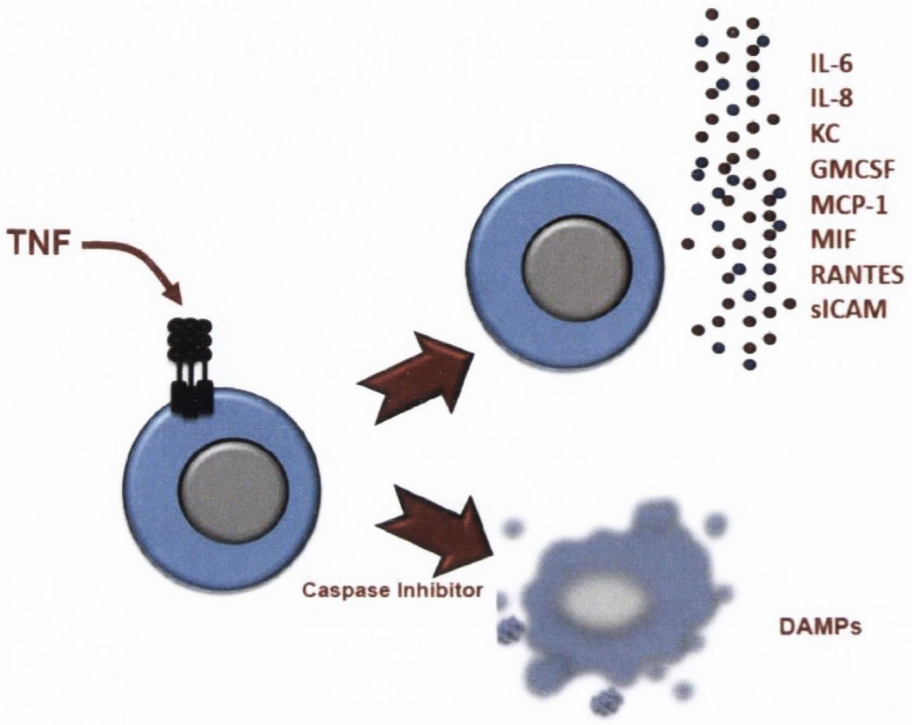

Figure 4.1

Necroptosis results in release of DAMPs but terminates TNF-induced cytokines, which outcome is more inflammatory? TNF stimulation does not normally result in cell death, but results in secretion of multiple pro-inflammatory cytokines and chemokins. If caspase-8 activity is blocked, TNF stimulation can result in rapid necroptosis. Rapid necroptosis results in a cessation in cytokine production but DAMPs are liberated. It is unclear at present which outcome is more inflammatory, an issue we now address.

and report that this mode of cell death resulted in a dramatic suppression of the production of multiple TNF-induced cytokines and chemokines, which was not compensated by the release of intracellular DAMPs. Knockdown of RIPK3 inhibited TNF-induced necroptosis and restored pro-inflammatory cytokine production. Thus, intracellular pathogens encoding caspase inhibitors that skew TNF or LPS signaling towards necroptosis may do so as an adaptive strategy to suppress the host inflammatory response.

4.2 RESULTS

4.2.1 TNF induces the production of multiple pro-inflammatory cytokines and chemokines regardless of cell death

While TNF is often considered a pro-apoptotic factor, this cytokine is predominantly an initiator of inflammation that is capable of transcriptionally upregulating a battery of inflammatory gene expression events, in the absence of cell death. Indeed, the majority of cell types typically do not undergo apoptosis in response to TNF unless transcription or translation is inhibited (Beg and Baltimore, 1996; Van Antwerp et al., 1996). To illustrate this, we conducted gene expression array analyses on HeLa cells, which revealed dramatic up-regulation of numerous pro-inflammatory cytokines and chemokines in response to TNF (Figure 4.2), in the complete absence of cell death (Figure 4.3). We confirmed protein secretion of the up-regulated genes (Figure 4.3) Thus, TNF is highly pro-inflammatory whether cells die or not.

Murine L929 cells have been widely used as a model system to explore the mechanism of TNF-induced programmed necrosis as they undergo necroptosis in response to TNF alone, which can be dramatically increased by zVAD (i.e. TNF-induced cell death that occurs where caspases are inhibited). However, the impact of necroptosis on TNF-induced inflammatory events has not been explored to date. This is surprising because TNF-induced necroptosis is frequently considered to be more pro-inflammatory than TNF-initiated outcomes that occur in the presence of caspase

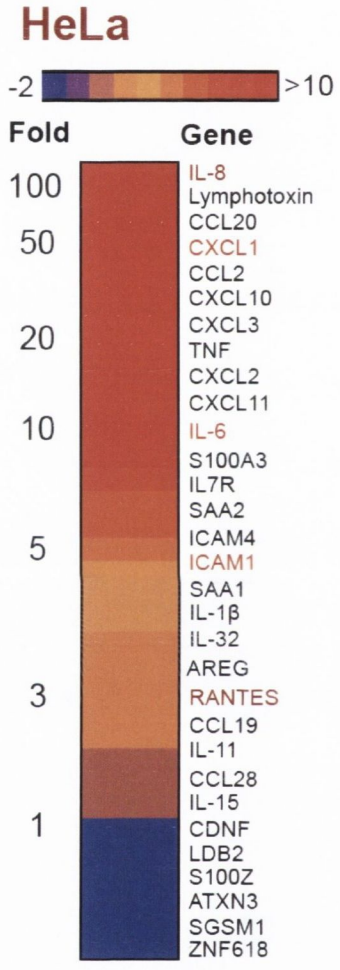

Figure 4. 2

TNF induces a potent pro-inflammatory transcription program. HeLa cells either left untreated or were treated with TNF (10 ng/ml). After 6 h, cells were harvested and subject to full genome microarray analysis. Fold change represents a comparison of untreated with TNF. The heat map displays a selection of inflammatory mediators that were up-regulated in response to TNF.

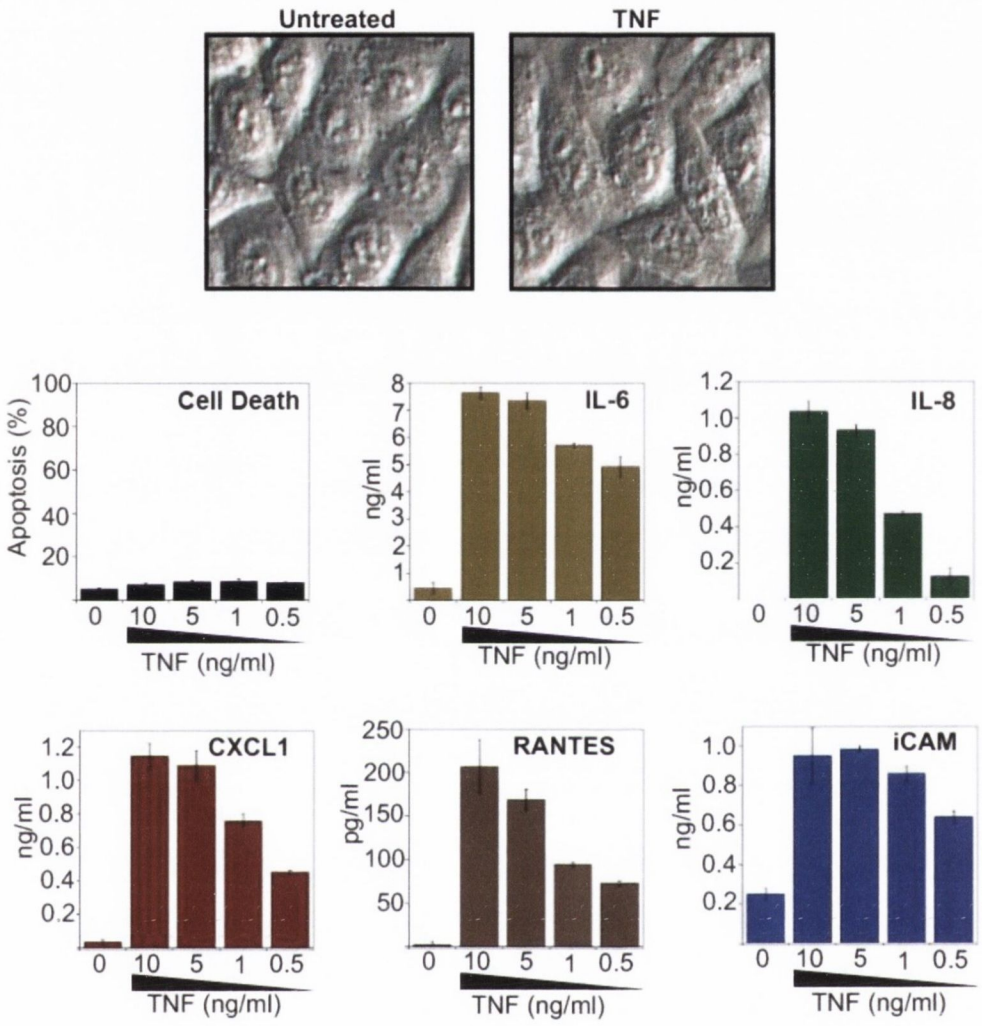

Figure 4. 3

TNF induces secretion of cytokines and chemokines in the absence of cell death. HeLa cells were either left untreated, or were stimulated with TNF at the indicated concentrations. After 24 h, apoptosis was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA. Representative phase contrast images are displayed, upper panel. A minimum of 300 cells were counted per treatment. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

activity (Duprez et al., 2011; Moriwaki and Chan, 2013; Vanlangenakker et al., 2012). This view appears to be predicated upon the notion that TNF promotes either apoptosis or necrosis, with the latter typically being pro-inflammatory and the former not. However, because TNF is itself highly pro-inflammatory as illustrated above (Figure 4.2 and 4.3), the impact of necroptosis on TNF-induced inflammatory events remains unclear. As shown in Figure 4.4, TNF-stimulation of L929 cells also resulted in the rapid synthesis of an array of cytokines and chemokines with 8 h of treatment, including: MCP-1, KC/CXCL1, MIP-2, GMCSF and IL-6. Indeed, large amounts of cytokines were detected prior to onset of considerable necroptosis.

4.2.2 Caspase inhibition accelerates RIPK3-dependant necroptosis, which is attenuated by Necrostatin-1

As has been demonstrated by many groups, TNFR stimulation in RIPK3-epressing cells can be switched to necroptosis through inhibition of caspase activity (Lin et al., 2004; Vercammen et al., 1998). As can be seen from Figure 4.5 *A, B and D,* the poly-caspase inhibitor zVAD-fmk resulted in much greater levels of TNF-induced necroptosis than TNF stimulation alone. We confirmed this dramatic acceleration of necroptosis in the presence of zVAD over a timecourse of TNF treatment (Figure 4.5 C). TNF/zVAD-induced necroptosis was inhibited by the addition of Necrostatin-1 (Figure 4.6 A), or through knockdown of RIPK3 (Figure 4.6 B and C). Similarly, MEFs also underwent necroptosis in response to TNF/zVAD treatment (Figure 4.6), which was also RIPK3-dependent and inhibited by Necrostatin-1 (Figure 4.6 D-F).

4.2.3 TNF-induced necroptosis inhibits the production of cytokines and chemokines

We next examined the impact of TNF-induced necroptosis on the release of TNF-induced pro-inflammatory mediators. As can be seen from Figure 4.7, whereas TNF treatment of L929 cells resulted in dose-dependent production of high concentrations of cytokines and chemokines over 24 h, addition of zVAD-fmk dramatically suppressed the production of MCP-1, KC, MIP-2, RANTES and GMCSF. As we found that MEFs also underwent necroptosis in response to

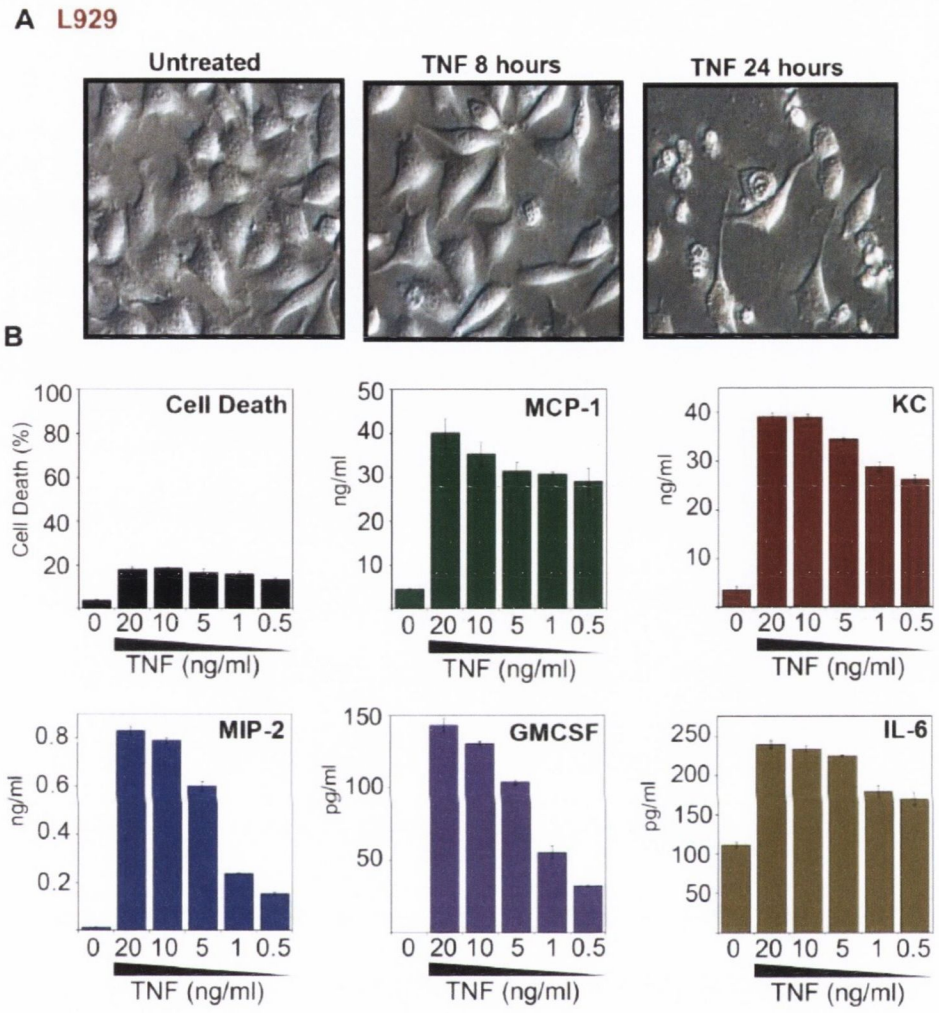

Figure 4.4

TNF induces secretion of cytokines and chemokines regardless of cell death **(A)** L929 cells were either left untreated, or were stimulated with TNF at the indicated concentrations. At the indicated time points, cells were visualized by phase contrast microscopy. **(B)** L929 cells were either left untreated, or were stimulated with TNF at the indicated concentrations. After 8 h, cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA. A minimum of 300 cells were counted per treatment. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

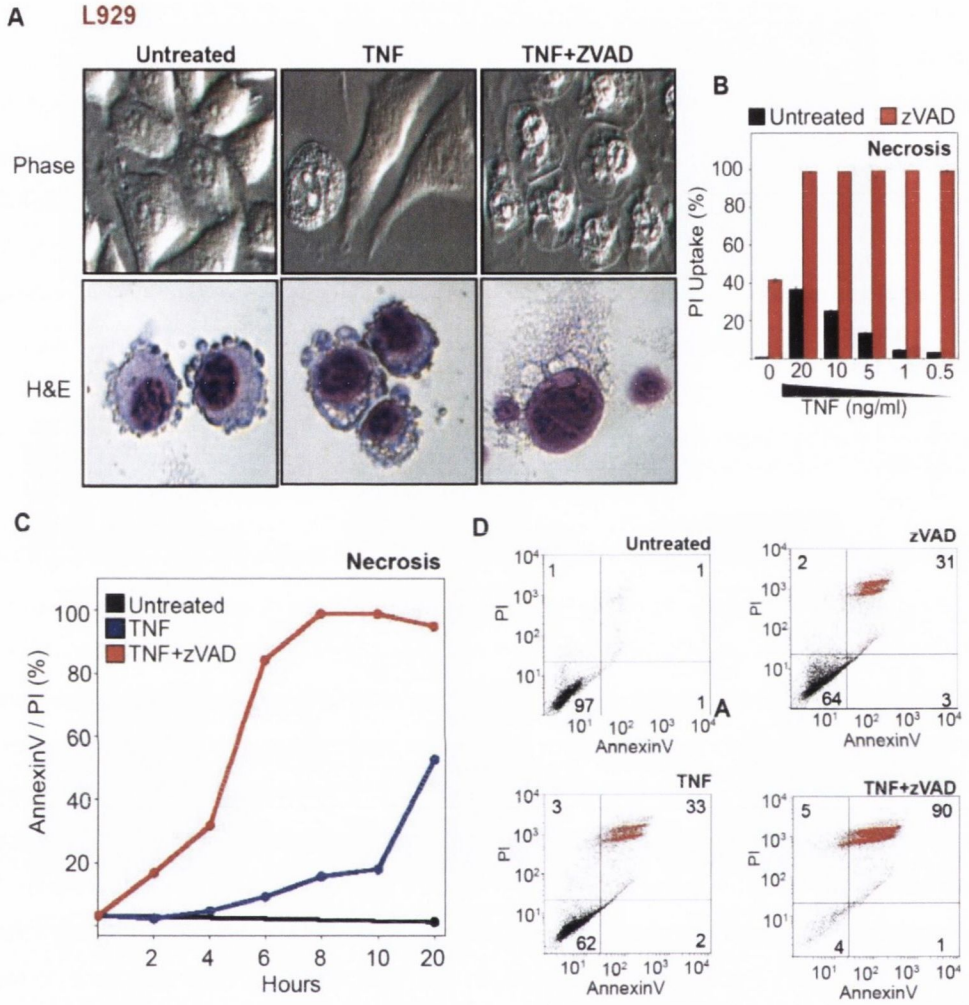

Figure 4.5

zVAD accelerates TNF- induced necroptosis. (A) L929 cells were either left untreated or treated with zVAD (10 μM) for 1 h, followed by stimulation with TNF (10 ng/ml). After 8 h, cells were visualised by phase contrast microscopy or by H&E staining on cytospins. **(B)** L929 cells were either left untreated, or treated with zVAD (10 μM) for 1 h, followed by stimulation with TNF (10 ng/ml). After a further 24 h, cell death was analysed by propidium iodide staining. **(C)** L929 cells were either left untreated, or treated with zVAD (10 μM) for 1 h, followed by stimulation with TNF (10 ng/ml). At the indicated time points, cell death was analysed by AnnexinV/ propidium iodide staining. **(D)** L929 cells were either left untreated, or treated with zVAD (10 μM) for 1 h, followed by stimulation with TNF (10 ng/ml). After a further 24 h, cell death was analysed by propidium iodide staining, representative flow cytometry charts are shown.

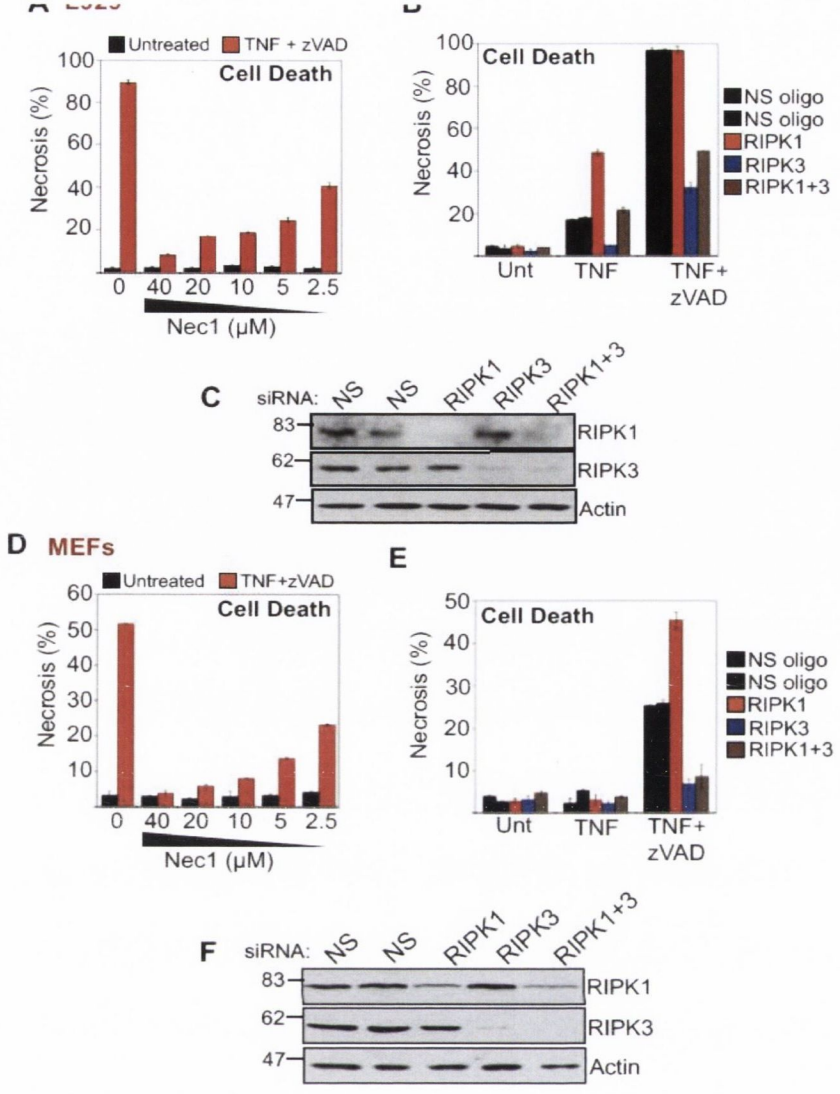

Figure 4.6

TNF induces RIPK3 dependant necroptosis that is inhibited by Necrostatin-1. **(A)** L929 cells were either left untreated, or treated with the indicated concentrations of necrostatin-1 for 1 h, followed by addition of TNF (10 ng/ml) and zVAD (10 μM). After 24 h, cell death was scored by morphology. **(B)** L929 cells were electroporated with the indicated siRNAs. Fourty eight hours later, cells were either left untreated, or treated with zVAD (20 μM) for 1 h, followed by addition of TNF (10 ng/ml). After a further 5 h, cell death was scored by morphological assessment. **(C)** Expression levels of RIPK1 and RIPK3 from *B,* were analysed by immunoblotting, 48 h after electroporation. **(D)** MEFs were either left untreated, or treated with the indicated concentrations of necrostatin-1 for 1 h, followed by addition of TNF (40 ng/ml) and zVAD (25 μM). After 24 h, cell death was scored by morphology. **(E)** MEFs were electroporated with the indicated siRNAs. Fourty eight hours later, cells were either left untreated, or treated with zVAD (25 μM) for 1 h, followed by addition of TNF (40 ng/ml). After a further 5 h, cell death was scored by morphological assessment. **(F)** Expression levels of RIPK1 and RIPK3 from *E,* were analysed by immunoblotting, 48 h after electroporation. A minimum of 300 cells were counted per treatment.

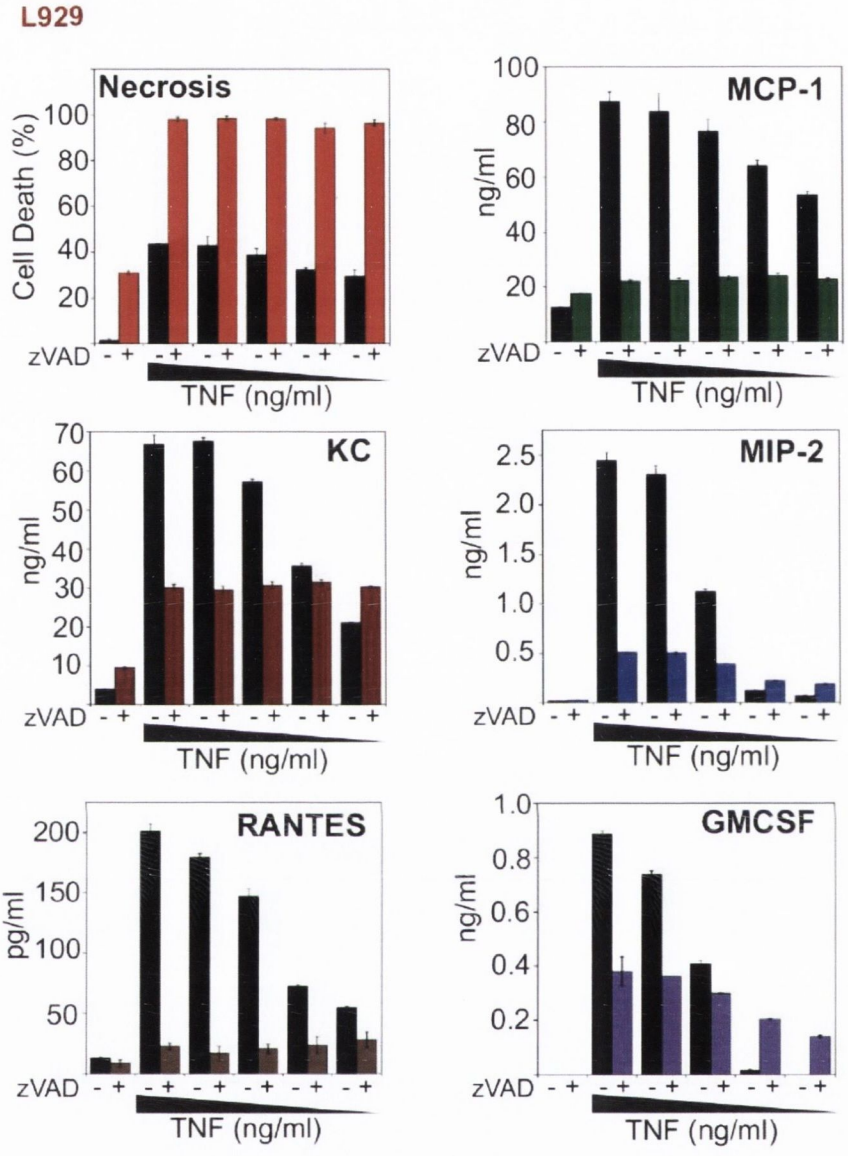

Figure 4.7

Necroptosis suppresses TNF-induced cytokine and chemokine production from L929 cells. L929 cells were either left untreated, or treated with zVAD (20 μM) for 1 h, followed by addition of TNF to a final concentration of 20, 10, 5, 1 or 0.5 ng/ml (from left to right). After a further 24 h, cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment. A minimum of 300 cells were counted per treatment.

TNF/zVAD treatment (Figure 4.6), we wondered would this also blunt TNF- induced cytokines, similarly to what we had observed in L929 cells. Indeed, we observed a similar dramatic reduction in TNF-induced pro-inflammatory cytokine production upon inducing necroptosis in MEFs (Figure 4.8).

Importantly, concentrations of zVAD that did not efficiently evoke necroptosis, did not result in cytokine suppression (Figure 4.9). We confirmed this result in MEFs also (Figure 4.10). Thus, the production of multiple TNF-induced cytokines and chemokines was suppressed as a consequence of triggering necroptosis, not due to an effect attributable to zVAD alone.

A timecourse analysis of cytokine production in response to TNF or TNF/zVAD-fmk again revealed a dramatic suppression of cytokine production during necroptosis, which correlated with much earlier cell death under necroptotic conditions (Figure 4.11). Some cells types are resistant to necroptosis under conditions of caspase inhibition alone, but can be sensitized by neutralizing IAPs or inhibiting transcription/translation in tandem with caspase inhibition (Figure 4.12). In this context, we also found that inducing necroptosis of HT29 cells using TNF/zVAD in the presence of IAP antagonist also dramatically suppressed cytokine production (4.12).

4.2.4 Caspase activity is not required for optimal TNF-induced cytokines and chemokines

Although we only observed suppression of TNF-induced cytokine production when necroptosis occurred, it was formally possible that zVAD was having a direct inhibitory affect on TNF-induced cytokine production. We had already ruled out a role for caspase activity in regulating TNF-induced cytokines in chapter one, however in this context it was very important to comprehensively exclude the possibility that zVAD had a direct inhibitory effect on TNF-induced cytokine production, without recourse to necroptosis. Initially we used HeLa cells as these

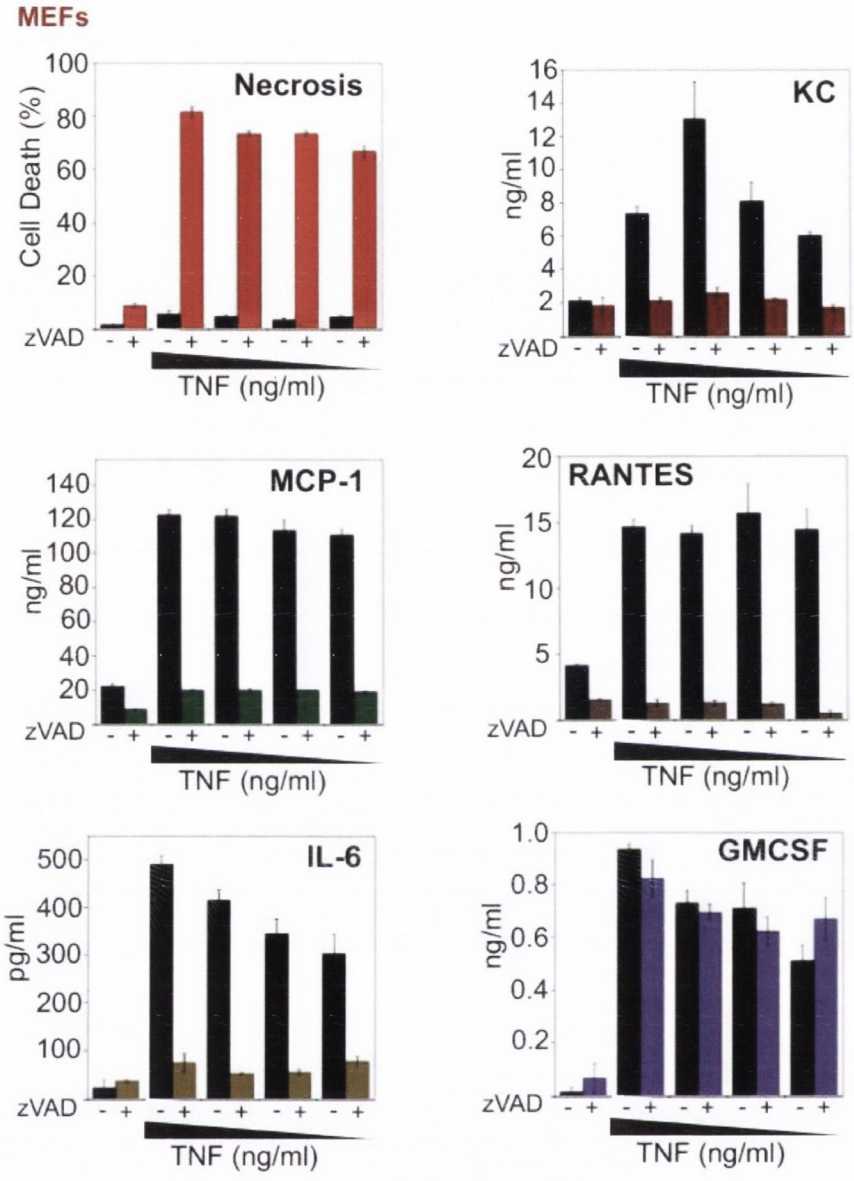

Figure 4.8

Necroptosis suppresses TNF-induced cytokine and chemokine production from MEFs. MEFs were either left untreated, or treated with zVAD (25 μM) for 1 h, followed by addition of TNF to a final concentration of 100, 50, 25, 12 or 6 ng/ml (from left to right). After a further 24 h, cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment. A minimum of 300 cells were counted per treatment.

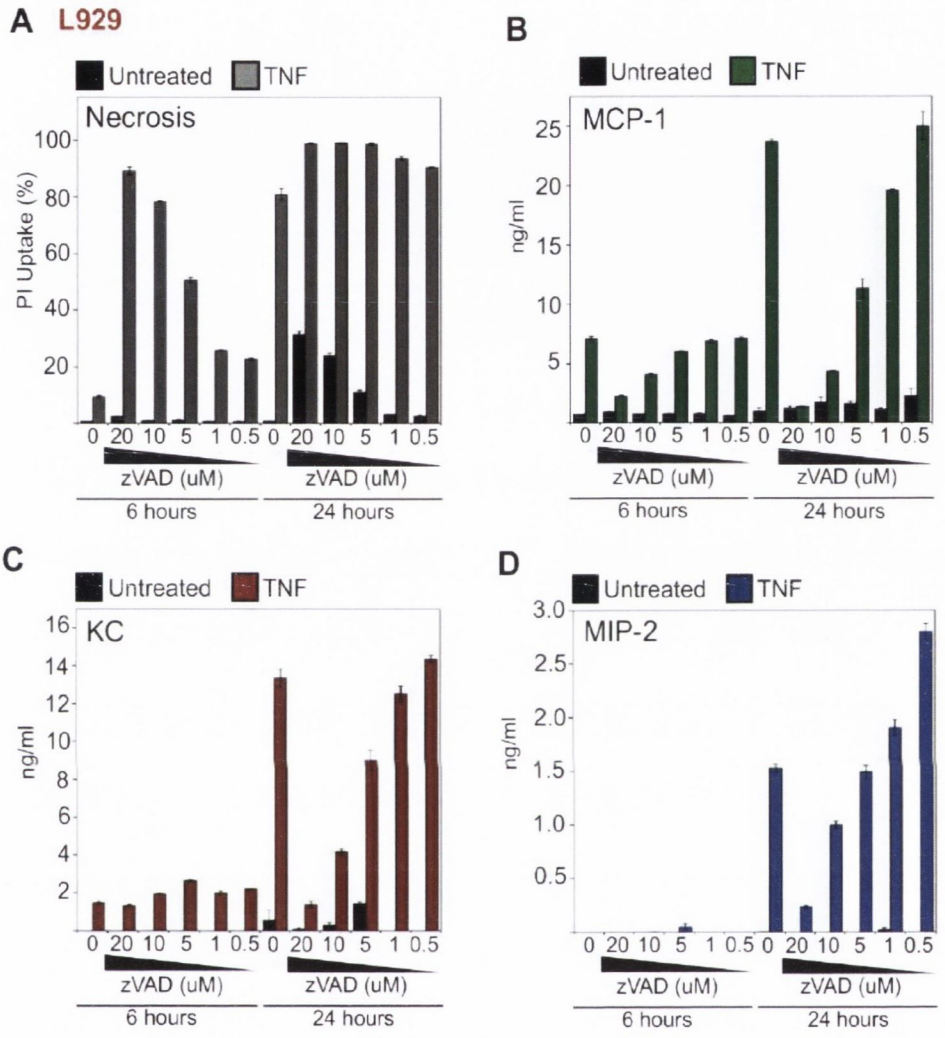

Figure 4.9

zVAD only inhibits cytokines at concentrations that efficiently evoke necroptosis of L929 cells. (A) L929 cells were left untreated, or treated with the indicated concentrations of zVAD for 1 h, followed by addition of TNF (10 ng/ml). After 6 and 24 h, cell death was analysed by propidium iodide staining and the culture supernatants were harvested to measure chemokines by ELISA **(B-D)**. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

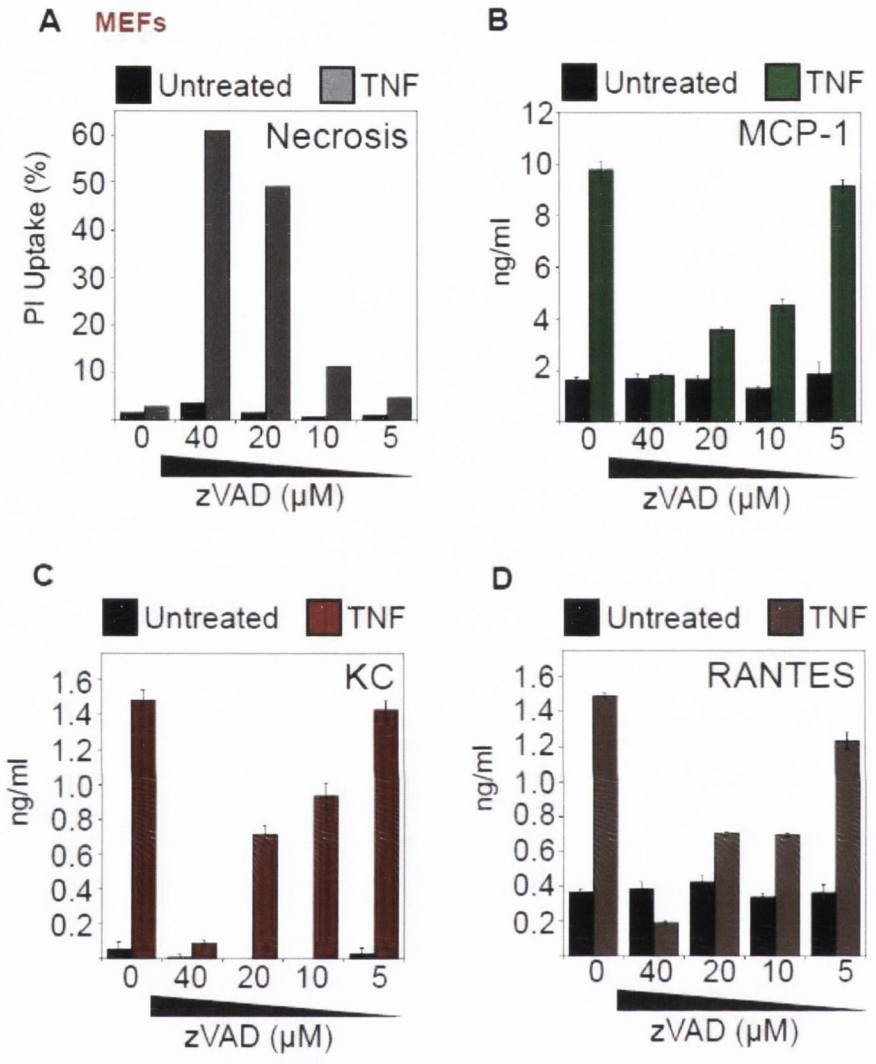

Figure 4.10

zVAD only inhibits cytokines at concentrations that efficiently evoke necroptosis of MEFs. (A) MEFs were left untreated, or treated with the indicated concentrations of zVAD for 1 h, followed by addition of TNF (10 ng/ml). After 24 h, cell death was analysed by propidium iodide staining and the culture supernatants were harvested to measure chemokines by ELISA **(B-D)**. Error bars represent the mean ±SEM of triplicate determinations from)a representative experiment.

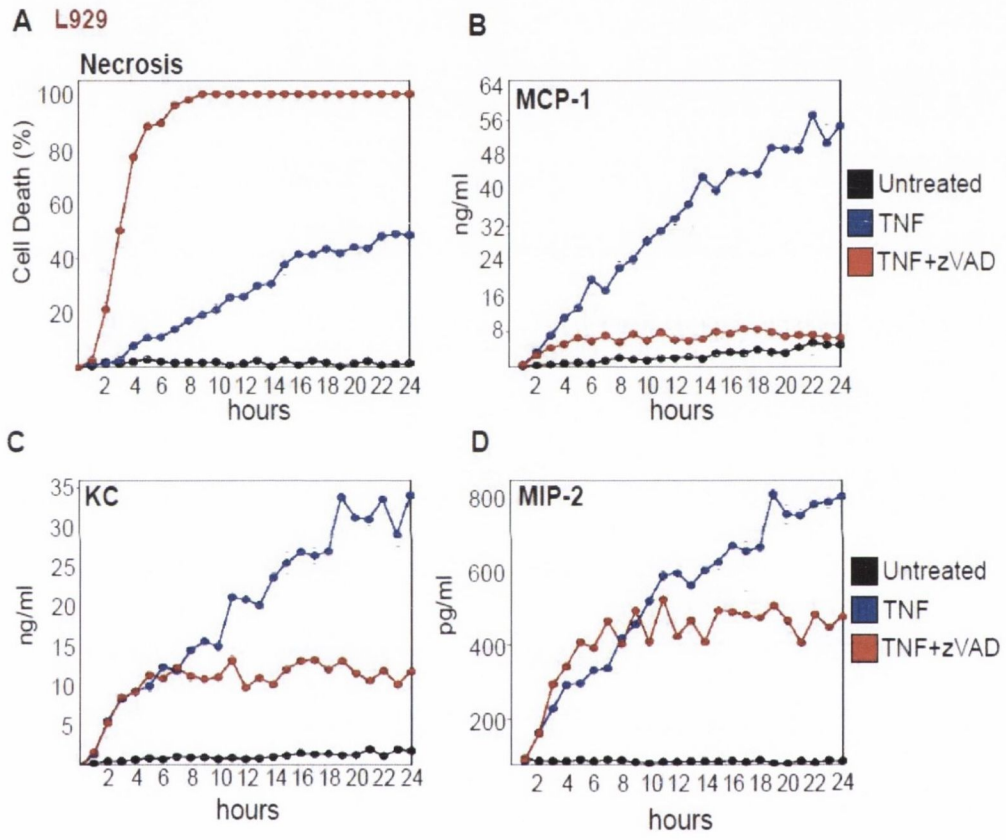

Figure 4.11

TNF- induced chemokine secretion is attenuated at the onset of necroptosis. **(A)** L929 cells were left untreated, or treated with zVAD (20 μM) for 1 h, followed by addition of TNF (10 ng/ml). At the indicated time-points, cell death was scored by morphology and the culture supernatants were harvested to measure chemokines by ELISA **(B-D)**. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment. A minimum of 300 cells was counted per treatment.

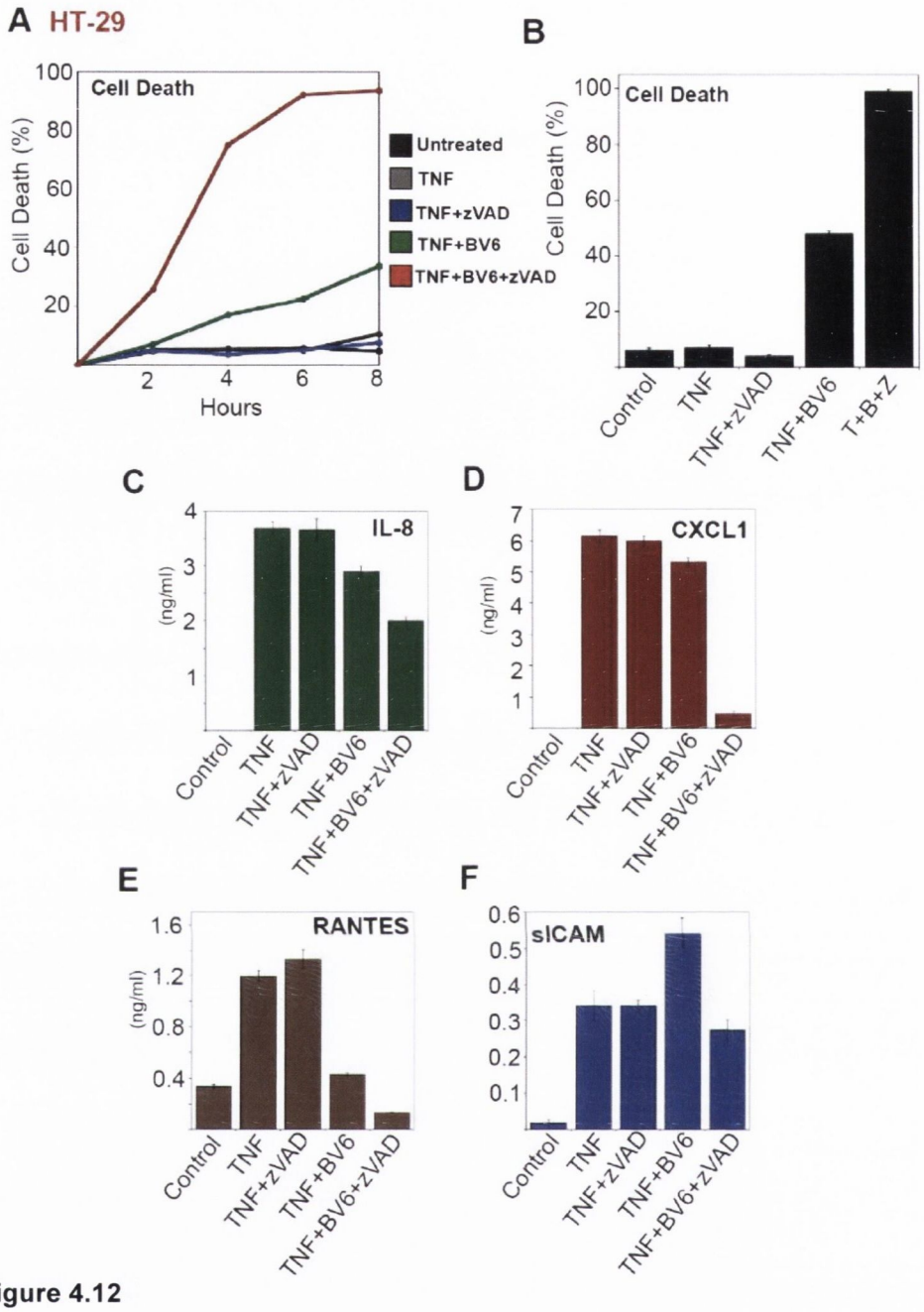

Figure 4.12

TNF-induced necroptosis suppresses inflammation in cells that require IAP antagonist- mediated sensitization. (A) HT-29 cells were either left untreated (control) or treated with BV6 (2μM) and/or zVAD (10 μM), for 1 h, followed by addition of TNF (10 ng/ml). at the indicated time points, cell death was analysed by morphology. **(B)** HT-29 cells were left untreated (control) or treated with BV6 (2μM) and/or zVAD (10μM), for 1 h, followed by addition of TNF (10ng/ml). After a further 24 h, cell death was analysed by morphology and cytokine/chemokine concentration in the supernatants was measured by ELISA **(C-F)**. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment. A minimum of 300 cells was counted per treatment.

cells lack RIPK3 and thus fail to undergo necroptosis in response to TNF/zVAD treatment (He et al., 2009). Treatment with zVAD efficiently blocked TRAIL-induced apoptosis in HeLa cells (Figure 4.13 A), but failed to cause necroptosis when combined with TNF (Figure 4.13 B). Under these conditions, zVAD failed to suppress TNF induced cytokine production (Figure 4.13 C-F), indicating that necroptosis is responsible for the suppression of cytokine production in RIPK3-expressing cells treated with TNF/zVAD. Furthermore, zVAD also potently inhibited Fas-induced apoptosis in HeLa cells (Figure 4.13 G) and consistent with this, Fas-induced IL-6 and KC were increased, rather than suppressed, in the presence of zVAD due to restoration of cell viability (Figure 4.13 H and I). Furthermore, zVAD failed to exert a suppressive affect on TNF-induced MCP-1 and KC in murine 3LL cells, which are also refractory to necroptosis (Figure 4.13 J-L). We also confirmed that zVAD did not inhibit TNF-induced cytokine synthesis at the mRNA level, in the absence of cell death (Figure 4.14).

4.2.5 Knockdown of RIPK3 or MLKL restores TNF-induced cytokine production

As noted above, a straightforward reason for necroptosis-mediated suppression of TNF-induced cytokine production was simply because cells underwent rapid cell lysis, thereby terminating cytokine production. However, an alternative reason could be that excessive recruitment of RIPK3 onto RIPK1, as a consequence of caspase inhibition, interfered with RIPK1-dependent recruitment of NEMO/IKKγ and NFκB activation downstream. Indeed, Dixit and colleagues have previously reported that RIPK3 suppresses RIPK1-dependent NFκB activation upon transient overexpression (Sun et al., 1999). Thus, it was formally possible that other effects of caspase inhibition (such as stabilization of the deubiquitinating enzyme CYLD), as opposed to RIPK3-dependent cell death, were responsible for suppressing TNF-induced inflammatory cytokine production.

To explore whether RIPK3-dependent cell death was the primary reason for the suppression of TNF-induced cytokine production under necroptosis-inducing

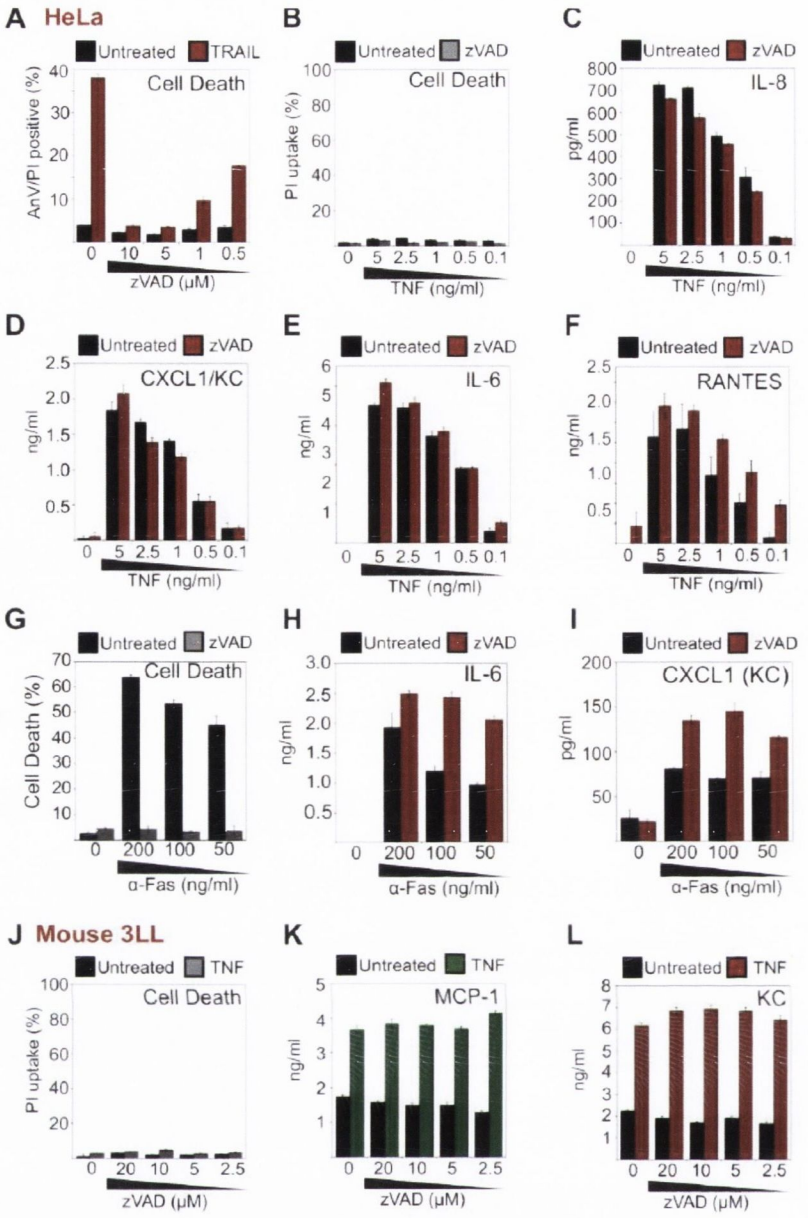

Figure 4.13

Caspase activity is not required for TNF-induced cytokine production. (A) HeLa cells were left untreated, or treated for 1 h with indicated concentrations of zVAD, followed by addition of TRAIL (100 ng/ml). After 8 hours, cell death was analysed by AnnexinV/Propidium Iodide staining. **(B-F)** HeLa cells were either left untreated or treated with zVAD (10 μM) for 1 h, followed by addition of TNF (10 ng/ml). After 24 h, cell death was analysed by AnnexinV/Propidium Iodide staining and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA. **(G-I)** HeLa cells were either left untreated, or with zVAD (10 μM), for 1 h, followed by addition of anti-Fas (CH11), (100 ng/ml). After 24 h, cell death was analysed by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA. **(J-L)** Murine 3LL cells were either left untreated, or treated with the indicated concentrations of zVAD for 1 h, followed by addition of TNF (10 ng/ml). After 24 h, cell death was analysed by Propidium Iodide staining and chemokine concentrations in the culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment. A minimum of 300 cells were counted per treatment.

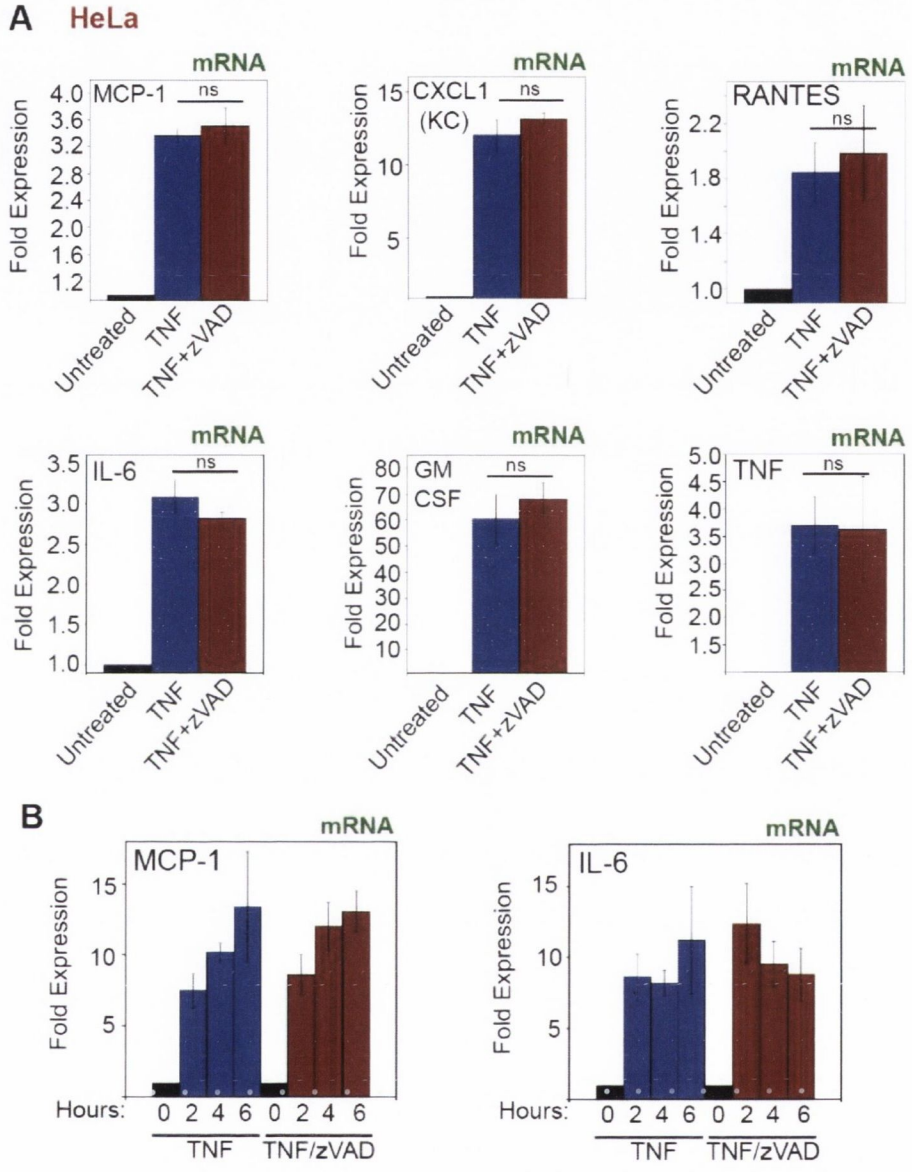

Figure 4.14

Caspase activity is not required for TNF-induced gene induction. (A) HeLa cells were either left untreated, or treated with zVAD (10 μM) for 1 h, followed by addition of TNF (10 ng/ml). After 4 hours, or the indicated time points **(B)**, total RNA was isolated, reverse transcribed and used to seed real-time PCR reactions. Error bars represent the mean ±SEM of triplicate cell cultures.

conditions, we silenced RIPK3 expression, followed by stimulation with TNF/zVAD to assess the effects on TNF-induced necroptosis and cytokine production. As shown in Figure 4.15 *A*, knockdown of RIPK3 blocked TNF-induced necroptosis and also partly restored TNF-induced cytokine production in the presence of zVAD (Figure 4.15 B-D). Similarly, knockdown of the downstream target of RIPK3, MLKL, also blocked TNF/zVAD-induced necroptosis (Figure 4.15 A) and restored cytokine production (Figure 4.15 B-D), suggesting termination of cell viability via RIPK3 and MLKL activation was responsible for the decline in cytokine synthesis.

We also examined cytokine and chemokine production within 5 h of stimulation of TNF alone, or TNF/zVAD, to ask whether cytokine production or NFκB activation upstream were attenuated prior to significant cell death. These experiments suggested that early TNF-induced cytokine production (Figure 4.16), as well as NFκB and MAPK activation (Figure 4.17), were highly similar in response to TNF and TNF/zVAD treatment, again suggesting that the decline in cytokine synthesis seen under necroptosis-inducing conditions was due to cell death rather than impaired signal transduction.

Because the RIPK1 inhibitor, Necrostatin-1, can block TNF/zVAD-induced necroptosis, we also used this approach to ask whether blocking cell death under necroptotic-inducing conditions restored cytokine production. Necrostatin-1 efficiently blocked TNF/zVAD-induced necroptosis, as expected (Figure 4.18). However, we also observed that Necrostatin-1 had robust inhibitory effects on TNF-induced KC and MIP-2 production, in the absence of zVAD (Figure 4.18). This suggests that at least some of the anti-inflammatory effects that have been attributed to Necrostatin-1 in previous studies could be due to direct effects on TNF-induced cytokine production, rather than due to inhibition of necroptosis. However, this aside, we also observed that Necrostatin-1 treatment did restore MCP-1 and KC production in response to TNF/zVAD-treatment to some degree (Figure 4.18). To investigate the effects of Necrostatin-1 on TNF-induced cytokines further, we also used MEFs which do not die in response to TNF alone (Figure 4.19 A). Once again, we observed that Nec1 significantly inhibited secretion of KC, RANTES and

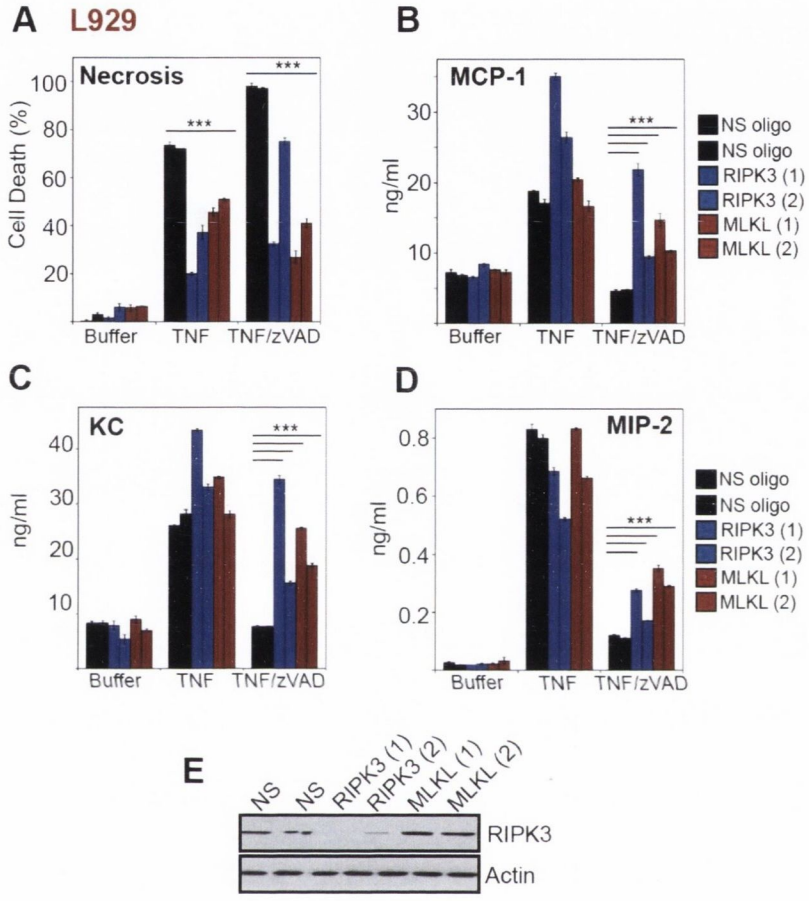

Figure 4.15

RIPK3 and MLKL-dependent termination of cell viability attenuates TNF-induced cytokine and chemokine production. (A) L929 cells were electroporated with the indicated siRNAs. Fourty eight hours later, cells were either left untreated (buffer), or treated with zVAD (10 μM) for 1 h, followed by addition of TNF (10 ng/ml). After 24 h, cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA **(B-D)**. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment. A minimum of 300 cells was counted per treatment. *p<0.1; **p<0.05; ***p<0.01 by Student's t test.

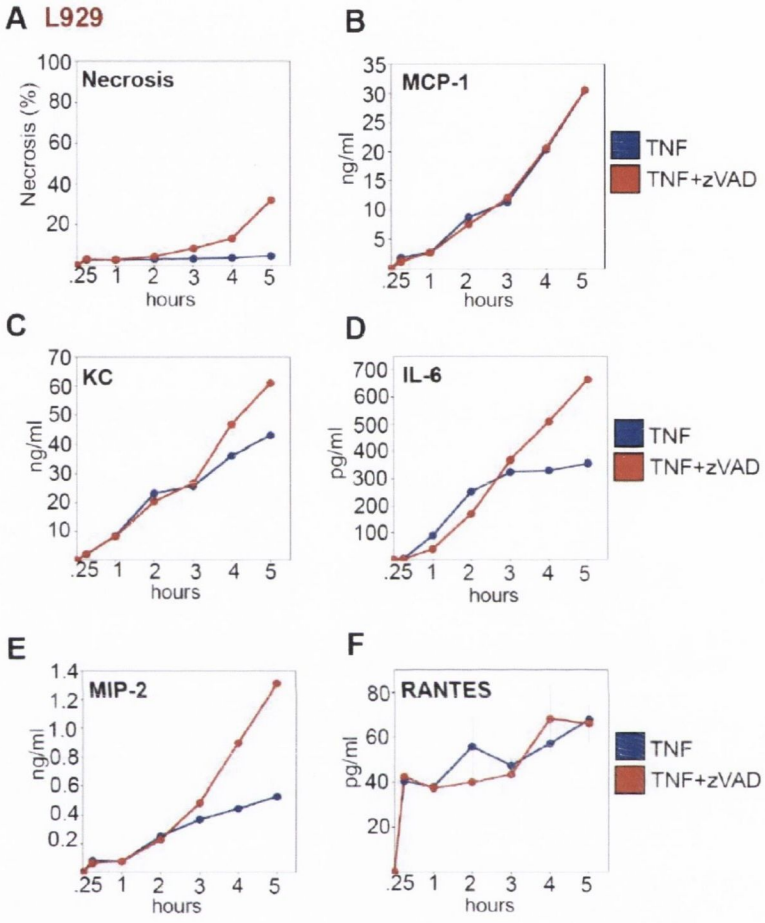

Figure 4.16

zVAD does not inhibit cytokine secretion prior to necroptosis. (A) L929 cells were either left untreated, or were treated with zVAD (10 μM) for 1 h, followed by addition of TNF (1 ng/ml). At the indicated time-points, cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA **(B-F)**. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment. A minimum of 300 cells was counted per treatment.

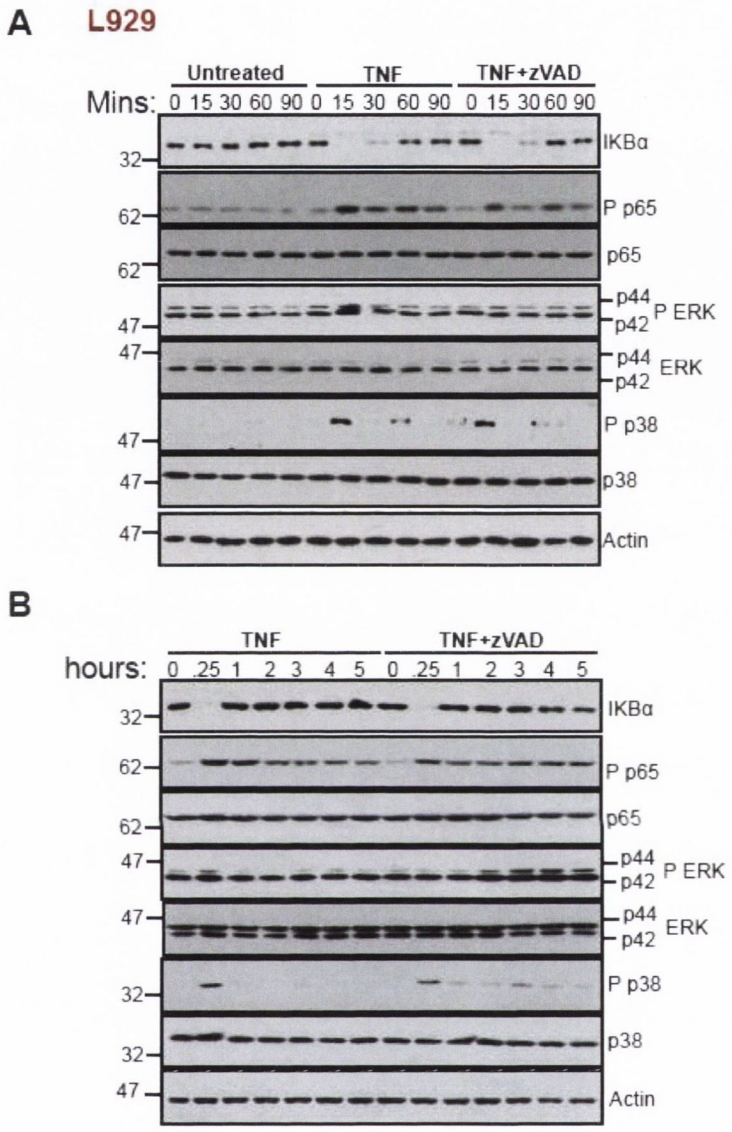

Figure 4.17

zVAD does not inhibit TNF-induced activation of NFκB or MAPKs. (A, B) L929 cells were left untreated, or were treated with zVAD (10 μM) for 1 h, followed by addition of TNF (1 ng/ml). Cell lysates were prepared at the indicated time points and analysed by immunoblotting for levels of endogenous proteins.

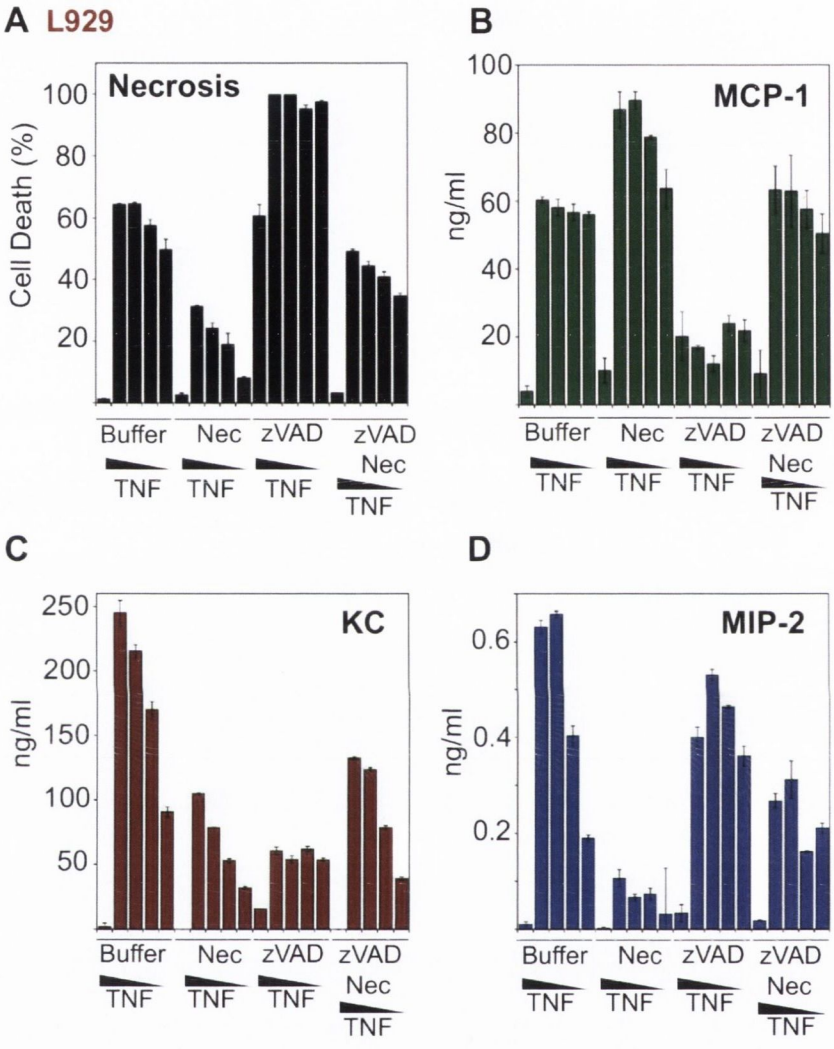

Figure 4.18

Inhibition of necroptosis with Necrostatin-1 partly restores cytokine production in cells treated with TNF / zVAD. (A) L929 cells were left untreated (buffer) or treated with necrorostatin-1 (5 μM) and/or zVAD (10 μM), followed by addition of TNF to 20, 10, 5 or 2.5 ng/ml (from left to right). After 24 h, cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA **(B-D)**. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment. A minimum of 300 cells were counted per treatment.

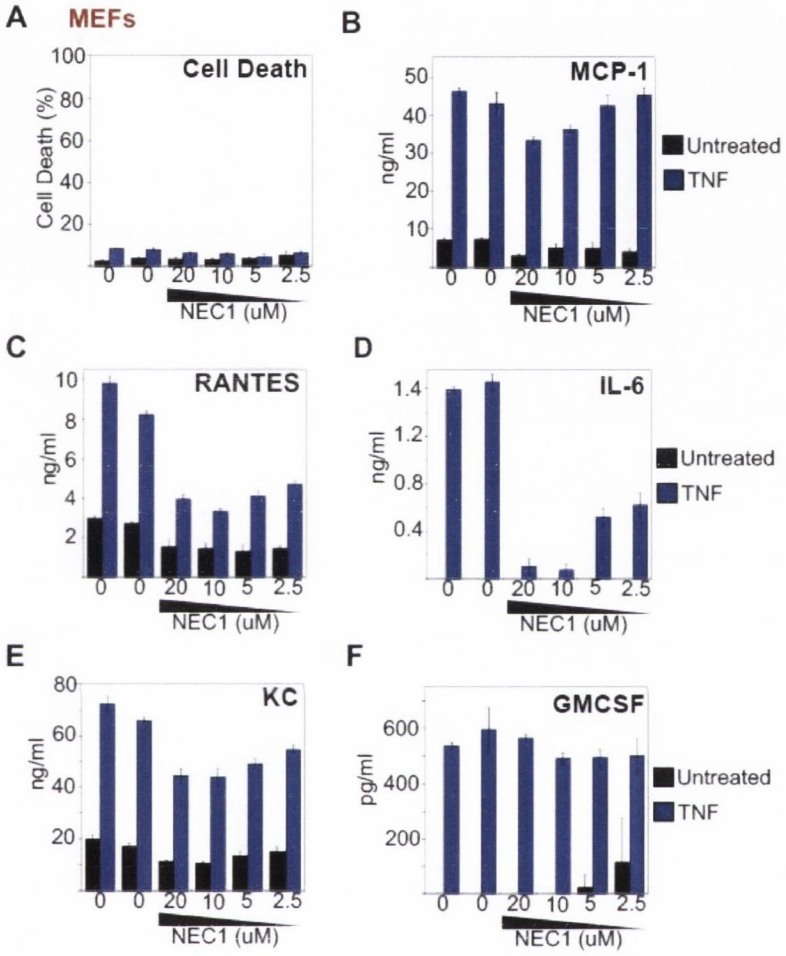

Figure 4.19

Necrostatin-1 inhibits TNF-induced cytokines in the absence of cell death. (A) MEFs were either left untreated, or treated with the indicated concentrations of Necrostatin-1 for 1 h, followed by addition of TNF (10ng/ml). After 24 h, cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA **(B-F)**. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment. A minimum of 300 cells were counted per treatment.

IL-6, but not GMCSF, in the absence of cell death or caspase inhibition (Figure 4.19 B-F).

4.2.6 Necroptosis suppresses LPS/TLR4-induced cytokine production

Because necroptosis can also be invoked in response to LPS stimulation, we next explored whether this also blunted pro-inflammatory cytokine and chemokine production in this context. As shown in Figure 4.20 A, LPS induced robust necrosis in primary bone marrow-derived macrophages (BMDM) in the presence of zVAD-fmk. LPS/zVAD-induced necroptosis was partly inhibited by addition of Necrostatin-1 (Figure 4.20 B) or through knockdown of RIPK3 (Figure 4.20 C). However, a component of the LPS-induced necrosis observed was not Necrostatin-1 inhibitable or blocked by RIPK3 knockdown. Moreover, similar to what we observed with TNF, LPS-induced IL-6, MIP-2, RANTES, KC, MCP-1 production were also considerably blunted under necroptosis-inducing conditions (Figure 4.21).

It is important to note that IL-1β, a key LPS-induced cytokine, requires caspase-1 activity, which is blocked under necroptosis-inducing conditions. Thus, LPS/zVAD-treatment greatly reduced the production of mature IL-1β as expected (Figure 4.22 B). Consistent with this, supernatants from LPS/zVAD-treated BMDMs had greatly reduced pro-inflammatory activity when transferred onto HeLa cells (See Figure 4.22 A) (as measured by the production of IL-8 from the latter), when compared with supernatants from BMDMs treated with LPS alone (Figure 4.22 C). Using MEFs, which do not engage RIPK3 or undergo necroptosis upon LPS/zVAD treatment, we also confirmed that zVAD did not suppress LPS-induced chemokines independently of necroptosis (Figure 4.23). Collectively, the above data indicate that necroptosis attenuates the production of numerous LPS-induced pro-inflammatory cytokines in RIPK3-expressing cells via termination of cell viability and through inhibition of caspase activity which is required for IL-1β maturation.

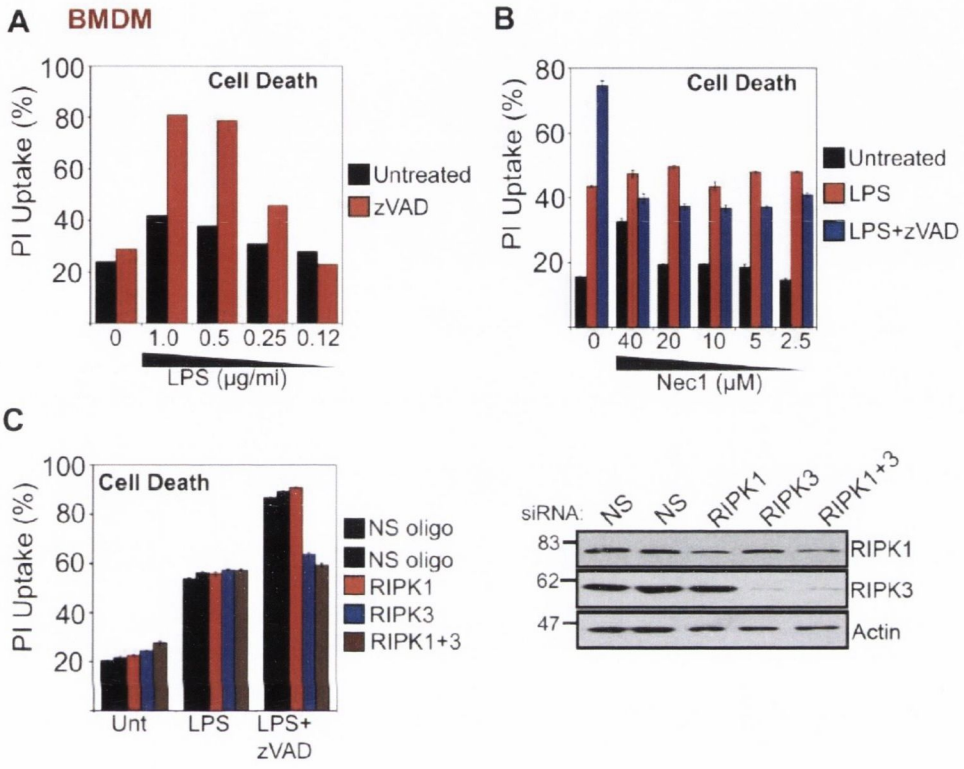

Figure 4.20

LPS induces RIPK3-dependant necroptosis of BMDMs in the presence of zVAD, which is inhibited by Necrostatin-1. (A) BMDMs were either left untreated, or treated with zVAD (15 μM), followed by addition of LPS to the indicated concentrations. 48 h later, cell death was analysed by propidium iodide staining. **(B)** BMDMs were either left untreated, or treated with the indicated concentrations of Necrostatin-1 in the presence or absence of zVAD (15 μM) for 1 h, followed by addition of LPS (1 μg/ml). After 24 h, cell death was scored by propidium iodide staining. **(C)** BMDMs were electroporated with the indicated siRNAs. Fourty eight hours later, cells were either left untreated, or treated with zVAD (15 μM) for 1 h, followed by addition of LPS (1 μg/ml). After 24 h, cell death was scored by propidium iodide staining. Expression levels of RIPK1 and RIPK3 were analysed by immunoblotting 48 h after electroporation, right panel.

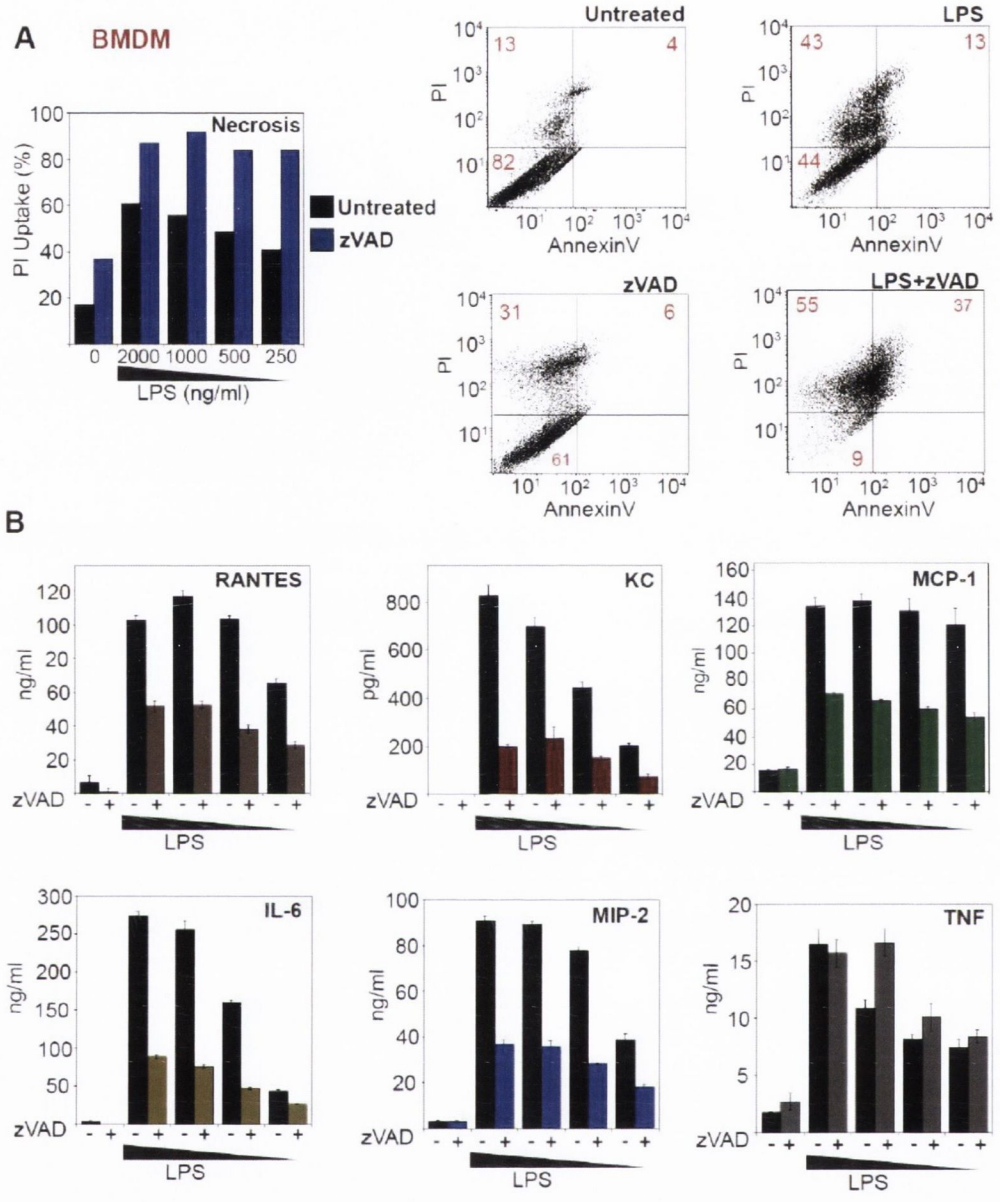

Figure 4.21

LPS induced necroptosis attenuates cytokine and chemokine production. (A) BMDMs were either left untreated, or treated with zVAD (15 μM) for 1 h, followed by addition of LPS to 2, 1, 0.5 and 0.25 μg/ml (left to right). After a further 48 h, cell death was analysed by propidium iodide staining. Representative flow cytometry charts are shown on the right. **(B)** Cytokine/chemokine concentrations in the culture supernatants from *A*, were determined by ELISA. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

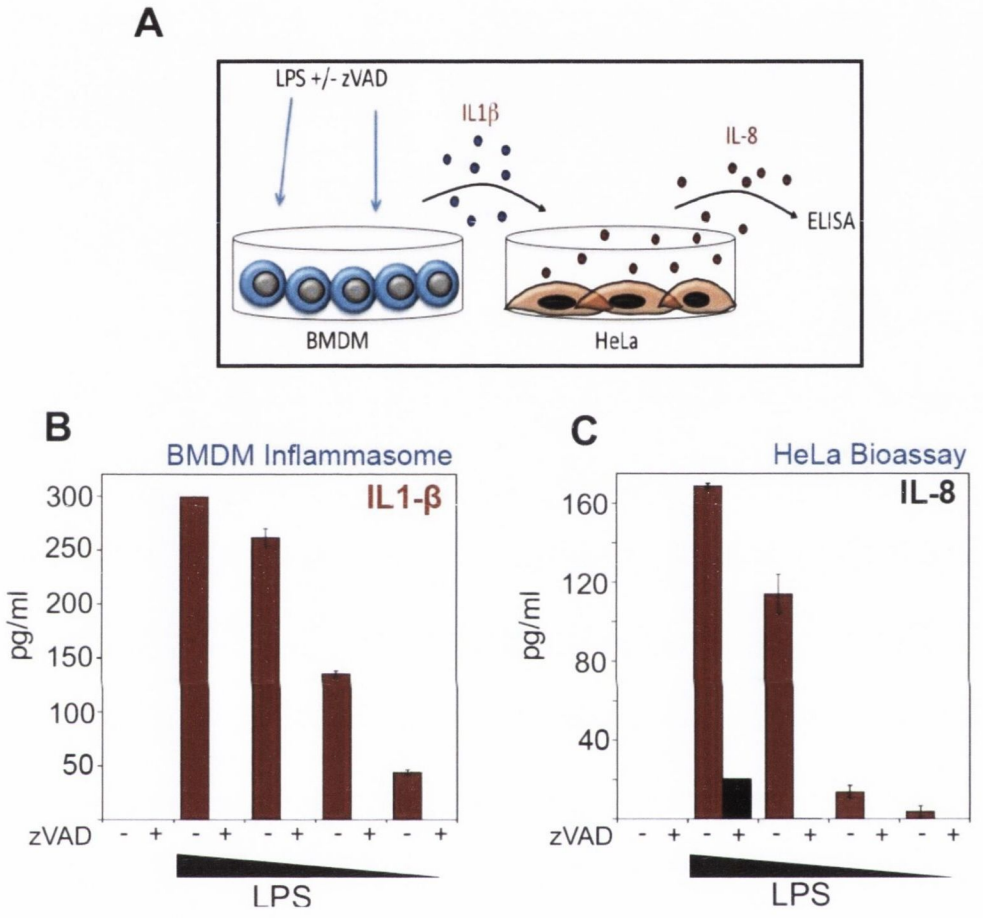

Figure 4.22

Necroptotic conditions prevent IL1-β maturation. (A) Schematic representation of the experiments described in *B* and *C*. **(B)** BMDMs were either left untreated, or treated with zVAD (15 μM) for 1 h, followed by addition of LPS to the indicated concentrations. 48 h later, culture supernatants were analysed for mature IL-1β by ELISA **(C)**. HeLa cell cultures were treated with 100 μl of the BMDM supernatent from *B*. After 24 h, HeLa supernatents were analysed for IL-8 by ELISA (HeLa bioassay). Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

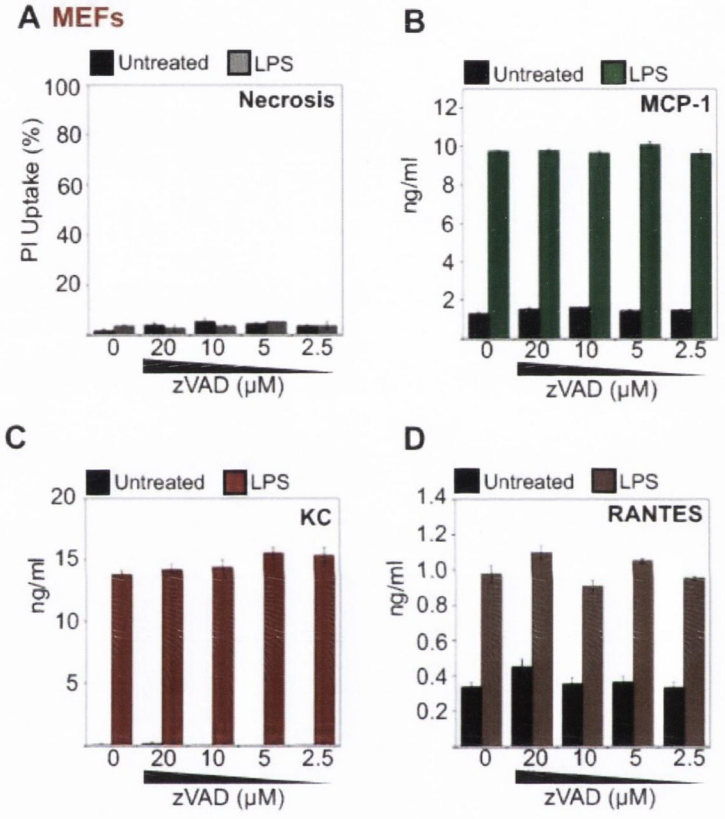

Figure 4.23

zVAD does not inhibit LPS- induced cytokines in the absence of cell death. (A) MEFs were either left untreated, or treated with the indicated concentrations of zVAD for 1 h, followed by addition of LPS (5 μg/ml). After 24 h, cell death was analysed by Propidium Iodide staining and chemokine concentrations in the culture supernatants were determined by ELISA **(B-D).** Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

4.2.7 TNF-induced necroptosis results in reduced inflammation *in vivo*

The preceding experiments demonstrated that TNF or LPS-induced cytokine and chemokine production was dramatically suppressed under necroptosis-inducing conditions. However, a caveat is that necroptosis liberates intracellular constituents, some of which can function as DAMPs, that could potentially compensate for the loss of cytokine and chemokine production. Thus, whether necroptosis enhances or suppresses TNF-induced inflammation depends on the relative potency of endogenous DAMPs, which are liberated via necroptosis, versus TNF-induced pro-inflammatory cytokines and chemokines in driving inflammatory processes *in vivo*.

To explore this issue, we introduced supernatants from L929 cells treated with TNF alone or TNF/zVAD (necroptotic) into the peritoneal cavities of immunocompetent BALB/c mice and monitored the peritoneal exudates after 24 h to explore whether supernatants from necroptotic cells were more or less inflammatory than those from cells treated with TNF alone (Figure 4.24). We generated supernatants from TNF-treated cells at high density, which contained much higher concentrations of pro-inflammatory cytokines than those from cells treated with TNF/zVAD, which rapidly died by necroptosis (Figure 4.25). Consistent with this, supernatants from TNF-treated cells induced robust infiltration of cells into the peritoneal cavities of mice (Figure 4.26 A), with increases in the percentages of neutrophils, lymphocytes, monocytes and small peritoneal macrophages (Figure 4.26 B and 4.27). In contrast, supernatants from TNF/zVAD-treated (necroptotic) cells exhibited little increase in peritoneal cellularity (Figure 4.26 A), or increases in neutrophils, lymphocytes or monocytes (figure 4.26 B, consistent with our *in vitro* observations. Thus, necroptosis attenuates rather than exacerbates TNF-induced inflammation.

To explore which chemokines were playing the dominant role in recruiting cells to the peritoneum in response to TNF-treatment (Figure 4.26 A), we also performed chemotaxis assays *ex vivo* using peritoneal exudate cells from TNF-treated mice. Chemotaxis of peritoneal exudate cells was measured in response to supernatants

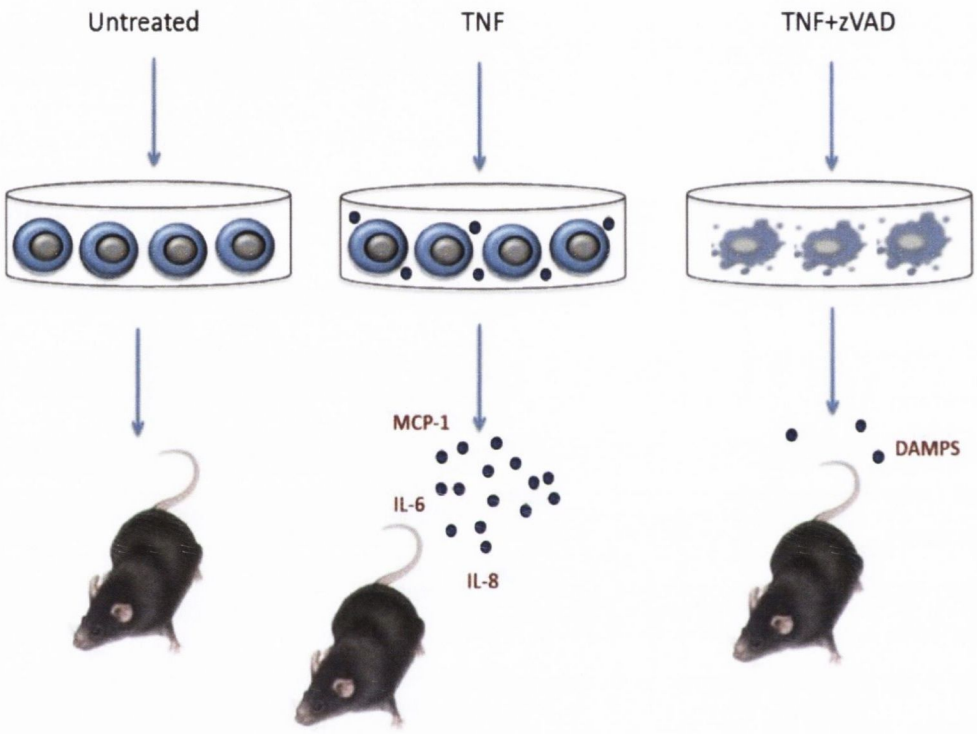

Figure 4.24

Schematic representation of experimental design to test the relative potency of conventional cytokines versus DAMPs, in vivo. High density ($2x10^6$) L929 cells were left untreated, or treated with zVAD for 1 h, followed by addition of TNF. After 24 h, the TNF treated cultures accumulated large amounts of cytokines, particularly MCP-1, while the TNF/zVAD treated cells died rapidly by necroptosis and liberated DAMPs. Supernatants from these conditions were then injected intraperitonealy into wild type mice to analyse their relative inflammatory potential.

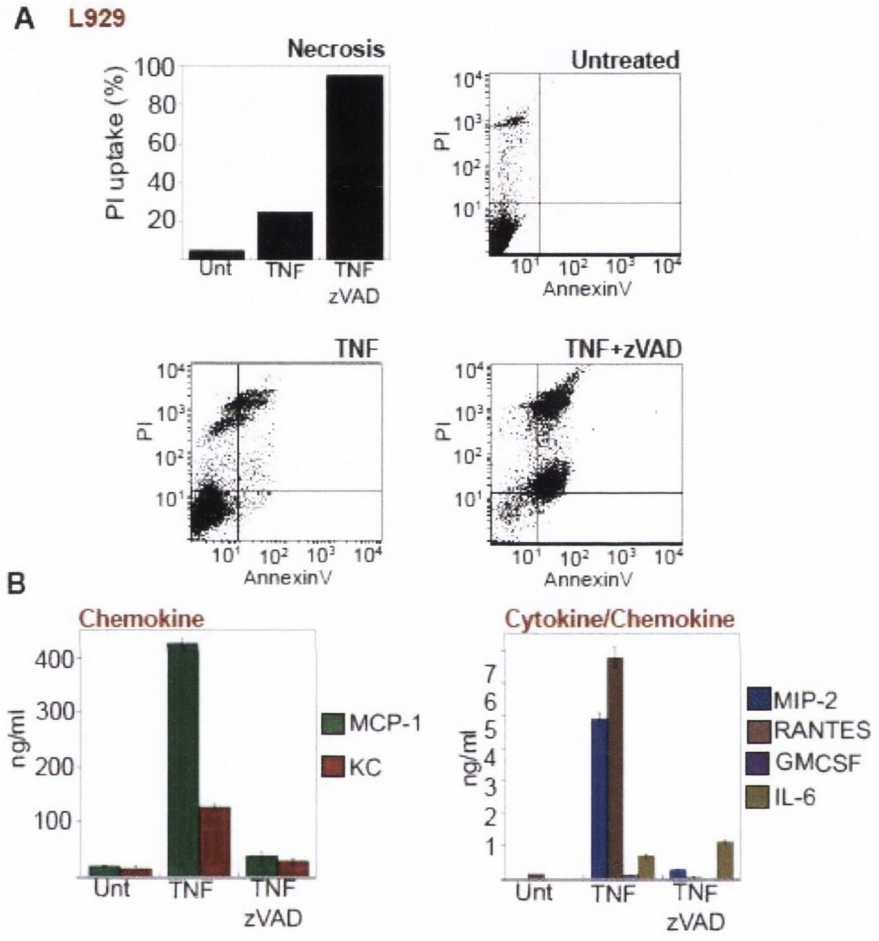

Figure 4.25

Generation of concentrated cell culture supernatants. (A) L929 cells at a density of $2x10^6$ per ml in RPMI supplemented with 1% FCS were either left untreated, or treated with zVAD (50 μM) for 1 h, followed by addition of TNF (10 ng/ml). After 24 h, cell death was analysed by propidium iodide staining and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA **(B)**. Supernatants were centrifugated to remove cells or debris.

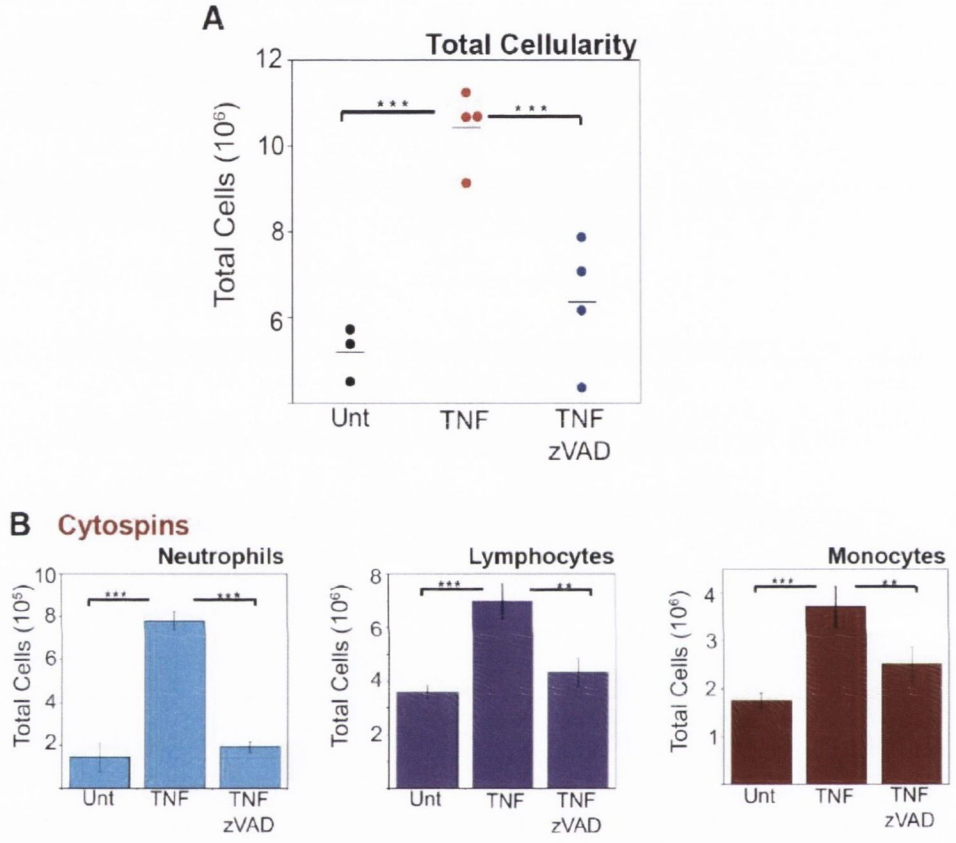

Figure 4.26

Necroptosis attenuates the inflammatory properties of TNF-stimulated cells in vivo. (A) Female Balb/c mice (4 per group) were injected intraperitoneally with 300 μl of the indicated L929 cell supernatants from *Figure 4.25*. After 24 h, mice were sacrificed and the peritoneal cavity was washed with 4 ml PBS. Total cellularity was scored by microscopy. **(B)** Cytospins were made from peritoneal-derived cells, stained with hematoxylin/eosin and used to score the percentage of immune cell types by morphology. Population numbers were then generated from total cellularity values obtained in *A*.

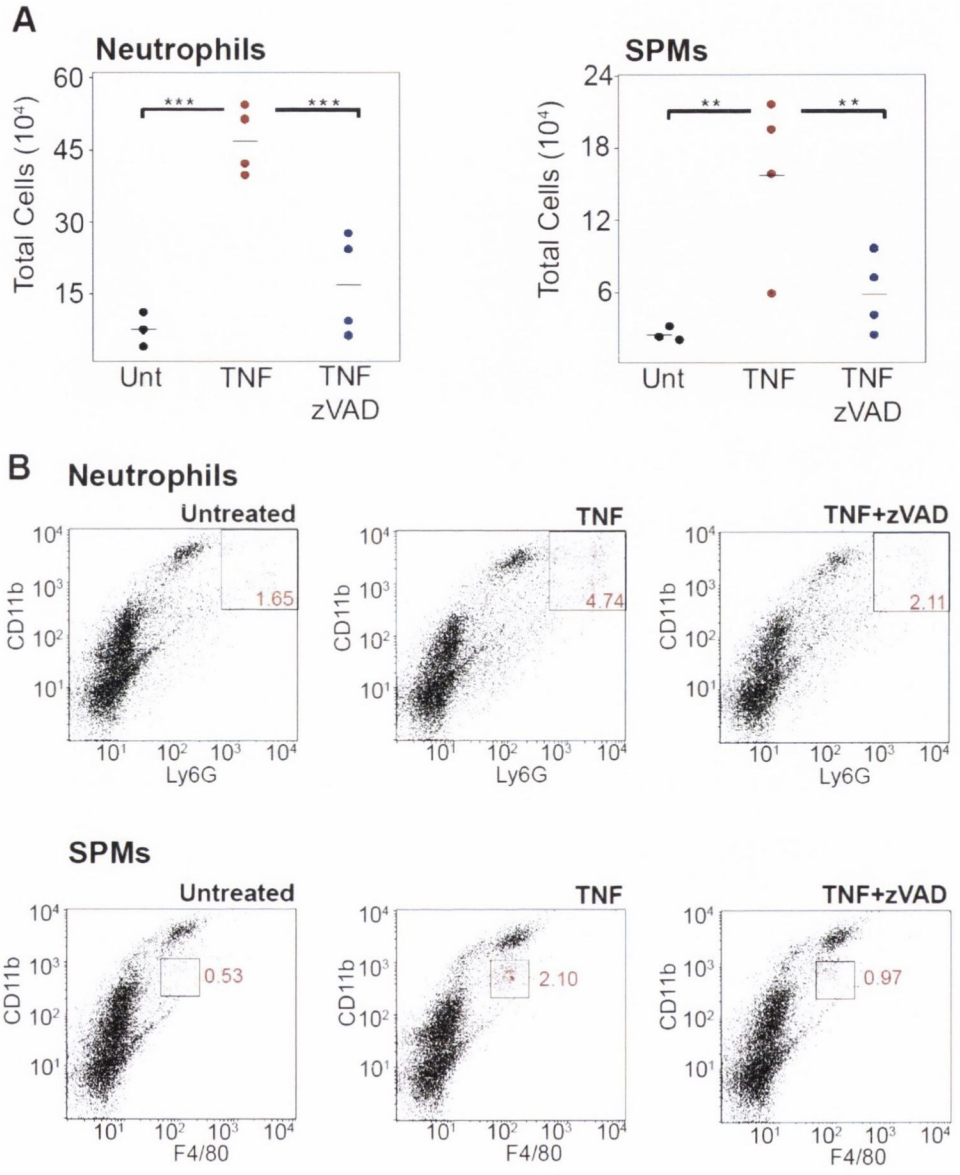

Figure 4.27

Necroptotic supernatents diminish recruitment of neutrophils and small peritoneal macrophages to the peritoneum. (A) Female Balb/c mice (4 per group) were injected intraperitoneally with 300 μl of the indicated L929 cell supernatants from *Figure 4.25*. After 24 h, mice were sacrificed and the peritoneal cavity was washed with 4 ml PBS. Peritoneal cells were immunostained as indicated and used to Peritoneal infiltrates were immunostained and quantified by flow cytometry as indicated.

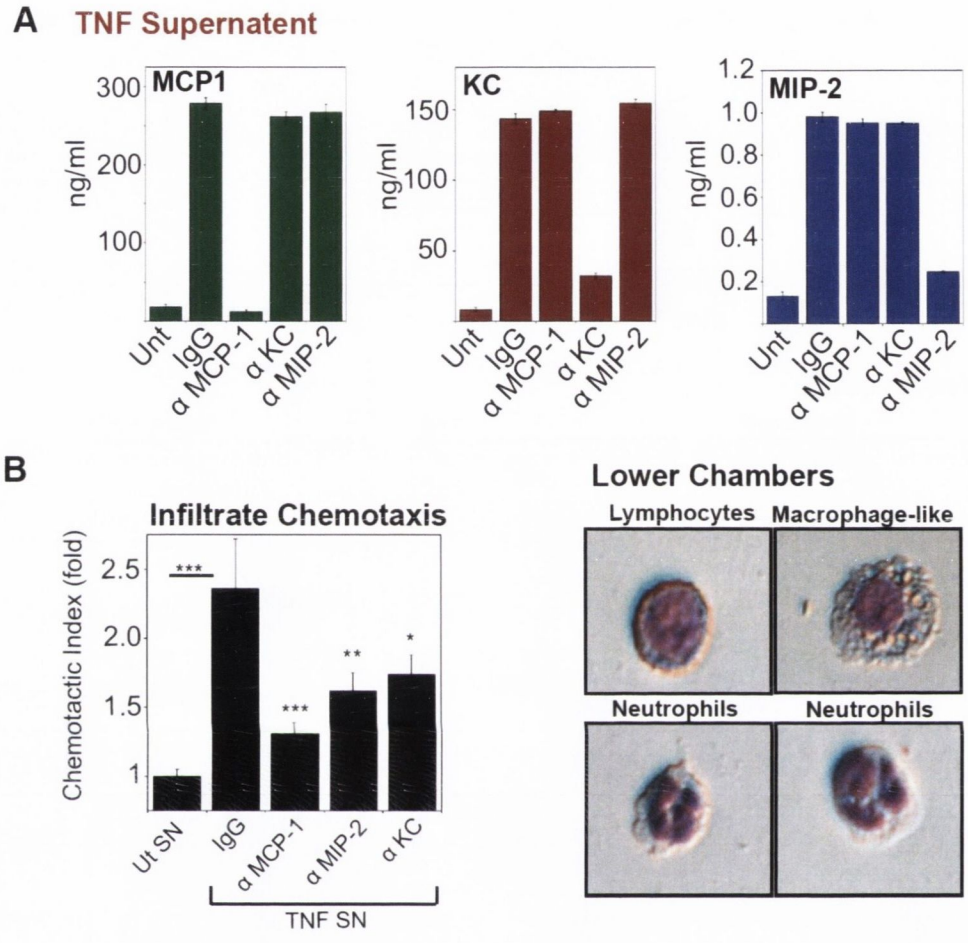

Figure 4.28

TNF-induced MCP-1 is a key factor in promoting recruitment of peritoneal-derived immune cells. (A) the supernatants from TNF treated L929 cells (figure 4.25), were immune-depleted of the indicated chemokines by coupling specific antibodies to A/G agarose beads. The depletion efficiency was analysed by ELISA. **(B)** Depleted supernatants from *A* were used in a chemotaxis assay using peritoneal infiltrates from mice that received supernatants from TNF-treated L929 cells. Cells from each individual mouse were used as experimental replicates (n=4). Cytospins were made from cells that successfully migrated, stained with H&E and viewed by phase contrast microscopy, right hand panel. Error bars represent the mean ±SEM *, $p<0.1$; **,$p<0.05$; ***,$p<0.01$ by students *t* test.

from TNF-treated cells that were either mock depleted (IgG), or depleted with anti-MCP-1, anti-CXCL-1, or anti-MIP-2 monoclonal antibodies (Figure 4.28 A). As shown in Figure 4.28 B, chemotaxis of peritoneal exudate cells was largely abolished upon depletion of MCP-1 from the supernatants, consistent with the very high concentrations of this chemokine produced in response to TNF (Figure 4.28 A).

4.2.8 LPS-induced inflammation is suppressed through caspase inhibition

As demonstrated earlier, LPS also promotes necroptosis in the presence of caspase inhibition (Figure 4.20), which led to suppression of the production of LPS-induced cytokines *in vitro* (Figure 4.21 and 4.22). To explore whether caspase inhibition also attenuated LPS-driven inflammation *in vivo*, we treated balb/c mice with LPS intraperitoneally in the presence or absence of zVAD-fmk, to promote necroptosis. Previous studies have shown that LPS induces cell death in the peritoneal cavity, as well as activation and efflux of resident macrophages and lymphocytes (He et al., 2011; Yang et al., 2007). The latter events are mirrored by an increase in splenic cellularity, composed of migrating neutrophils, macrophages, monocytes and clonally expanding lymphocytes (Cao et al., 2005; Yang et al., 2007).

As shown in Figure 4.29 *A*, LPS administration led to a dramatic reduction in peritoneal cellularity which was significantly inhibited in LPS/zVAD treated animals. However, it should also be noted that zVAD alone also led to a reduction in peritoneal cellularity. Consistent with this, we observed an increase in splenic cellularity in response to LPS which was attenuated in presence of zVAD (Figure 4.29 B). We also confirmed that LPS/zVAD treatment caused a significant increase in peritoneal cell death, compared to LPS alone (Figure 4.29 C). As shown in Figure 4.30, LPS-treatment induced robust increases in splenic neutrophils, F4/80+ macrophages, eosinophils, and B- and T-lymphocytes, which were all diminished in the presence of LPS/zVAD. This is consistent with a suppression rather than enhancement of LPS-induced inflammatory responses under necroptotic (caspase

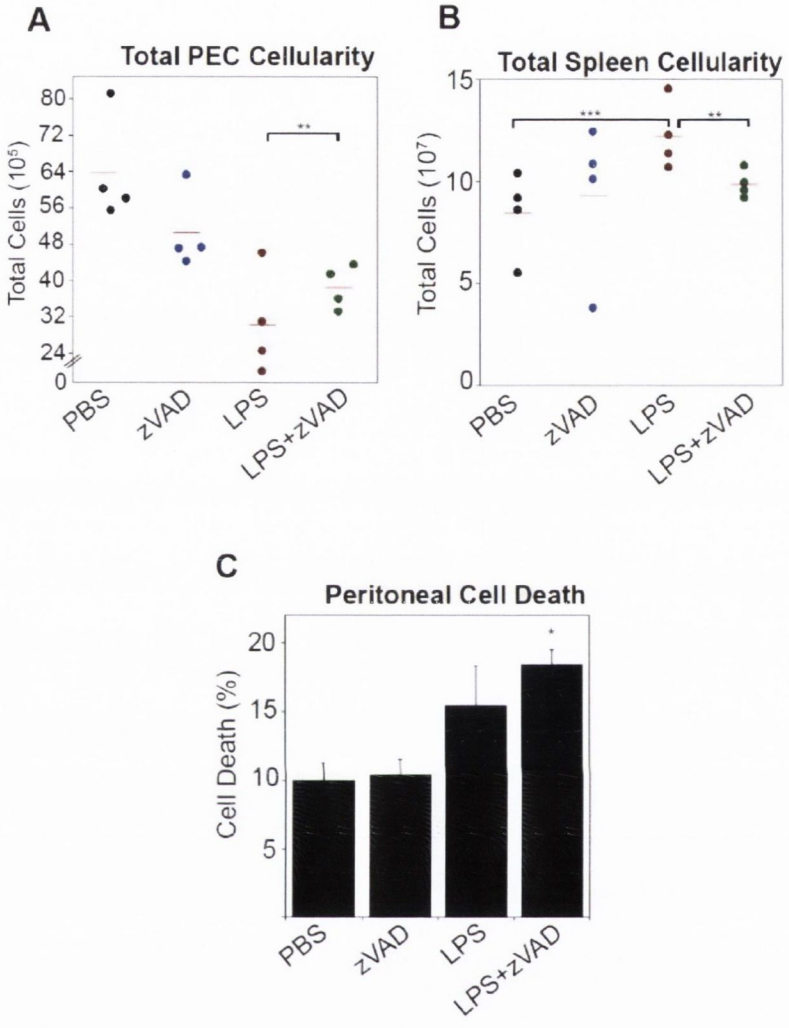

Figure 4.29

Necroptosis attenuates LPS-induced inflammation in vivo. **(A)** Female balb/c mice (4 per group) were injected (intraperitoneally, 200 μl) with PBS or zVAD (250 μg per mouse). After 1 h, mice were injected again with either PBS or LPS (10 μg per mouse). After a further 24 h, mice were sacrificed and the peritoneal cavity was washed with 4 mL PBS. Total cellularity was scored by microscopy. **(B)** Spleens were removed and splenocytes collected through a strainer. Erythrocytes were removed using RBC lysis buffer then total cellularity analyzed by microscopy. **(C)** Peritoneal cell death was analyzed by Aqua positivity. Error bars represent the mean ±SEM *, $p<0.1$; **,$p<0.05$; ***,$p<0.01$ by students *t* test.

inhibition) conditions. Once again, these data argue that necroptosis suppresses rather than enhances inflammatory responses, most likely through killing responding cells and reducing inflammatory cytokine production.

To explore this further, we recovered peritoneal cells from PBS-treated mice and stimulated with either LPS or LPS/zVAD to evaluate cytokine production *ex vivo*. LPS/zVAD-treated peritoneal exudates exhibited increases in cell death as compared to cells treated with LPS or zVAD alone (Figure 4.31 A). Furthermore, we again observed dramatic reductions in LPS-induced TNF, MCP-1, KC, MIP-2, and RANTES in the LPS/zVAD (necroptotic) condition (Figure 4.31 B).

Taken together, the above data argue that TNF- or LPS-induced necroptosis is significantly less pro-inflammatory than unimpeded TNF- or LPS-stimulation. Our data also argue that conventional TNF- or LPS-induced cytokines and chemokines exert more potent pro-inflammatory effects than endogenous DAMPs that are released during necrosis or necroptosis.

4.3 DISCUSSION

Here we have shown that, in contrast to current interpretations of the biological consequences of necroptosis, RIPK3-dependent cell death resulted in a dampening of TNF-induced pro-inflammatory events, thereby leading to a less inflammatory outcome. Similarly, we also found that LPS-initiated necroptosis was less inflammatory than LPS stimulation involving caspase participation. These data highlight the importance of considering the inflammatory consequences of TNF or LPS stimulation when switching the response to cell death, either pharmacologically or indeed genetically.

4.3.1 Conventional cytokines are more inflammatory that DAMPs

The necroptosis effectors RIPK3 and MLKL are likely to have other cellular

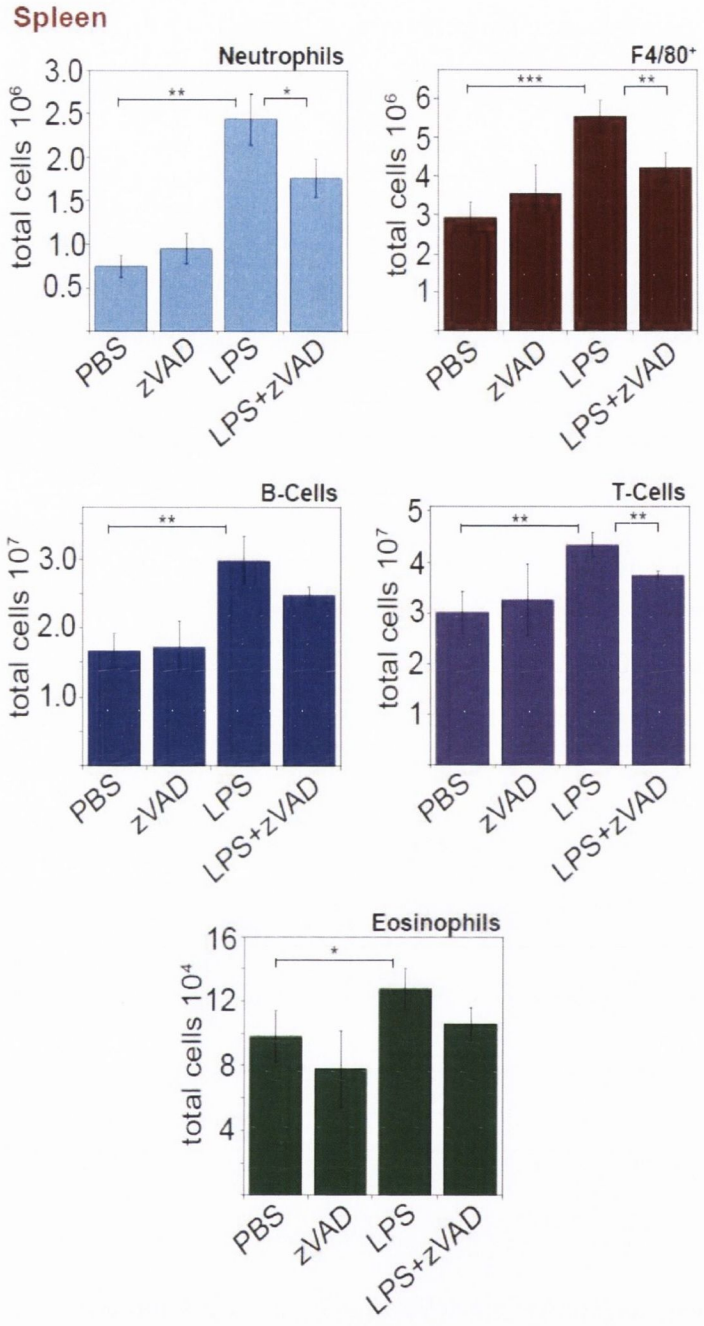

Figure 4.30

zVAD attenuates LPS-induced migration of immune cells to the spleen. Spleens were removed and splenocytes collected through a strainer. Erythrocytes were removed using RBC lysis buffer then splenocytes were immunostained and analysed by flow cytometry. Population numbers were then generated by multiplying cell percentages by total splenic cellularity counts. Neutrophils ($CD11b^+$, $Gr1^{hi}$, $F4/80^{med}$, $SiglecF^-$), Eosinophils ($SiglecF^+$), B-cells ($CD19^+$), T-cells ($CD3^+$). Error bars represent the mean ±SEM *, $p<0.1$; **,$p<0.05$; ***,$p<0.01$ by students *t* test.

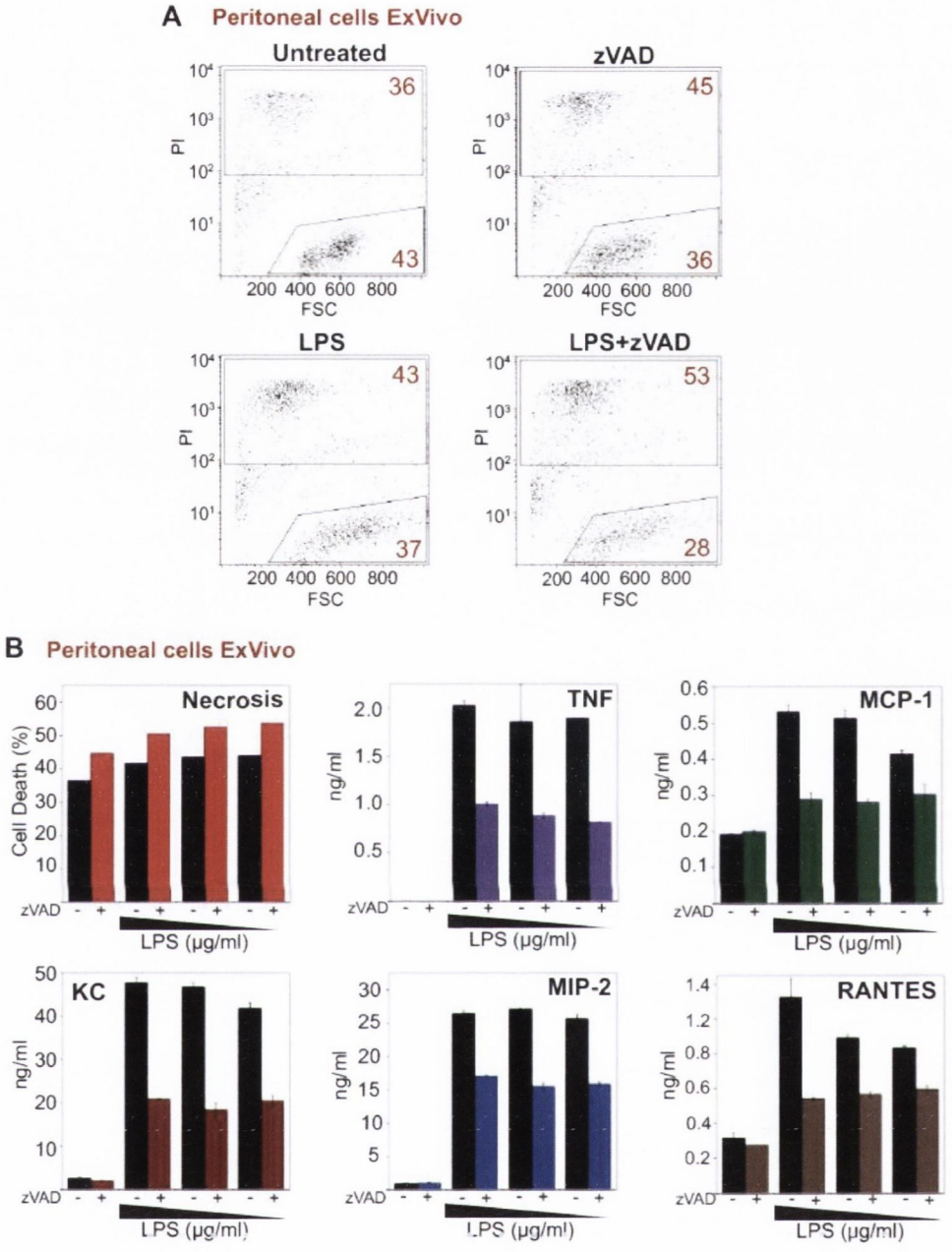

Figure 4.31

Necroptosis attenuates LPS-induced cytokine production from PECs ex vivo. **(A)** Peritoneal derived cells from control mice were seeded at $1x10^6$/ml in 24 well plates. Cells were either left untreated, or treated with zVAD (20 µM) for 1 h, followed by addition of LPS to 10, 5 and 1 µg/ml. After 24 h, cell death was analysed by forward scatter/propidium iodide staining (representative flow cytometry plots shown) and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA **(B).** Error bars represent the mean ±SEM of triplicate sample determinations.

functions, which remain unknown at present, but have evolved the capability to orchestrate programmed necrosis. However, we propose that these molecules could also be regarded as negative regulators of TNF- and LPS-induced inflammation, as their activation rapidly shuts down cytokine synthesis through rapid cell lysis. These findings have implications for the interpretation of the pathology of *RIPK3* null animals in response to pathogen challenge as well as sterile injury. By terminating cell viability, necroptosis leads to a dramatic cessation of inflammatory cytokine production and the liberation of endogenous DAMPs is insufficient to compensate for this. Therefore, proposed DAMPs such as DNA, ATP and HMGB1 (which are contained in all cells), do not trigger inflammation as potently as cytokines and chemokines. This finding indicates that conventional cytokines such as IL-36 family members, IL-33 and IL-1, which have TNF-like inflammatory potential, are likely to be the predominant DAMPs *in vivo*. These conventional cytokines qualify as DAMPs as they lack a leader sequence, thus require necrotic cell rupture for their release.

From these data, one could speculate that inflammatory conditions that occur in the absence of microbial infection (sterile inflammation), are driven predominantly by excessive cytokine production, not DAMPs. Thus, pathological conditions may arise from overproduction of cytokines which could potentially lead to cell destruction and DAMP release, causing a feedback loop. In this light, targeting of cytokines therapeutically is likely to result cessation of symptoms, as has been demonstrated with TNF-inhibitors.

4.3.2 Complications for the use of Necrostatin-1 *in vivo*

Support for our observations come from a study by Linkermann and colleagues, who reported that the necroptosis inhibitor, Necrostatin-1, failed to protect from TNF/zVAD-induced shock and lethality, but rather accelerated time to death compared to TNF/zVAD treatment alone (Linkermann et al., 2012a). This is consistent with our observations that necroptosis suppresses the inflammatory effects of TNF, which can lead to lethality, rather than enhancing these effects as is frequently speculated. Suppressing necroptosis in the context of TNF stimulation is likely to enhance the lethal effects of this cytokine through prolonging and exacerbating the cytokine storm. Moreover, Linkerman et al., also observed similar

effects with cerulein-induced pancreatitis, where Necrostatin-1 worsened the inflammatory symptoms, again most likely by restoring production of a plethora of TNF-inducible inflammatory mediators (Linkermann et al., 2012a). However, it should also be noted that interpreting effects seen with Necrostatin-1 *in vivo* is problematic, as Necrostatin-1 may have direct inhibitory effects on the production of some TNF-induced cytokines as we have shown. In particular, we have found that TNF-induced production of IL-6, which is a key player in models of severe systemic inflammation (SIRS) induced by TNF, is dramatically reduced in the presence of Necrostatin-1. Indeed, direct effects of Necrostatin-1 on TNF-induced cytokine production might well explain the protection afforded by this kinase inhibitor during TNF-induced shock *in vivo* (Duprez et al., 2011), especially as the latter effects were seen in the absence of caspase inhibition where necroptosis would not be expected.

4.3.3 Necroptosis may be a pathogen strategy to diminish cytokine/chemokine driven immunity

Our data cast doubt upon the view that necroptosis is invariably a host response to pathogens encoding caspase inhibitory proteins, as opposed to a pathogen-driven mechanism to limit the host inflammatory response. Our data favor the interpretation that infectious agents that promote necroptosis may do so as a mechanism to neutralize host immune responses. RIPK3 knockout mice display increased susceptibility to replication of vaccinia virus (Cho et al., 2009). This strongly implies that necroptosis is a method of combating this virus by depriving the virus of sufficient time to replicate. However, the role of necroptosis as an anti-viral strategy appears to be more complex and context dependent than initially presumed. Previous studies using poxviruses that encode caspase inhibitors have shown that virus deficient in CrmA, a potent Caspase-1 and -8 inhibitor is less pathogenic than wild type virus capable of inducing necroptosis and processing IL-1β (MacNeill et al., 2009). This suggests that the ability to inhibit caspase activity and trigger necroptosis can also be beneficial to the virus rather than the host in at least some situations. Similarly, rabbits infected with myxoma virus lacking the CrmA homolog Serp2 display increased viral control (Messud-Petit et al., 1998).

The preceding observations suggest that promoting necroptosis can be beneficial to viruses in certain contexts. This fits well with the implications of our observations and suggests that some viruses may deliberately shut down the host inflammatory response by invoking necroptosis in a cell that receives TNF stimulation. This might also have the consequence of facilitating viral spread by killing the very cells that act as sentinels in tissues vulnerable to viral infection. It is also relevant to note that were necroptosis an effective host response to viral infection, designed to terminate viral replication by depriving the virus of a host, then it would be expected that viruses would rapidly emerge that have lost their caspase inhibitory protein(s). However, the fact that multiple viruses do encode caspase inhibitors strongly suggests that these are beneficial to viral replication or spread. Thus, viral initiation of necroptosis could also be viewed a strategy adopted by certain viruses to suppress the host inflammatory response, rather than a host response designed to terminate viral replication.

4.3.4 Intracellular bacteria appear to benefit from host necroptosis

Infection with the intracellular bacterium *Salmonella Typhimurium* has also been shown to lead to RIPK3-dependent necroptosis (Robinson et al., 2012). However, infection of RIPK3 null macrophages (incapable of undergoing necroptosis) led to decreased bacterial burdens, once again suggesting that rapid RIPK3-dependent necroptosis of macrophages is beneficial to the pathogen and helps to attenuate the host immune response. Necroptosis may also permit escape of *Salmonella* into the extracellular space where it is known to be capable of undergoing replication. In support of this idea, Shayakhmetov and colleagues have recently demonstrated that *Listeria monocytogenes* triggers IRF-3-dependant macrophage necroptosis that facilitates bacterial infection while in the same model, necroptosis reduced disseminated infection with adenovirus (ADV) (Di Paolo et al., 2013). DAMP-driven Inflammation associated with macrophage necrosis could also be beneficial to pathogens by promoting recruitment of infection-susceptible monocytes and dendritic cells into the vicinity of extracellular bacteria. Thus, the consequences of necroptosis for pathogen survival may be context-dependent, with slow growing pathogens such as CMV and ADV blocking necroptosis as well as apoptosis to permit viral replication, but more rapidly replicating pathogens may exploit this cell

death pathway to suppress host immune responses and permit escape into the extracellular space.

Neutrophils are rapidly recruited from the peripheral circulation to sites of infection via chemokine gradients generated by tissue resident innate immune cells, where they deploy aggressive anti-microbial mechanisms. Local macrophages, which provide a rich source of neutrophil chemoattractants KC and MIP-2, are required for neutrophil migration into skin infected with *Staphyloccus aureus* and subsequent bacterial clearance (Abtin et al., 2014). However, *Staphyloccus aureus* utilizes virulence factor hemolysin to promote rapid macrophage necrosis at the infection site, thereby dampening chemokine generation and neutrophil recruitment (Abtin et al., 2014). This demonstrates the importance of the initial chemokine wave that is released in response to pathogen infection and highlights responder cell necrosis as a pathogen strategy to terminate it. Indeed, we also found that synthesis of chemokines KC and MIP-2 was rapidly terminated as a consequence of TNF- and LPS-induced necroptosis (Figure 4.7 and 4.21), and translated into impeded neutrophil recruitment to the peritoneal cavity (Figure 4.27 A) and spleen (Figure 4.30) *in vivo*.

In conclusion, we have provided evidence that necroptosis may be less inflammatory than the alternative outcomes (cell survival or apoptosis) due to rapid cessation the transcriptional pro-inflammatory program induced by TNF or LPS. Thus, viruses and other microorganisms that evoke this mode of cell death may well do so as an adaptive strategy to minimize the host inflammatory response.

CHAPTER 5

RIPK1 CAN FUNCTION AS AN INHIBITOR RATHER THAN AN INITIATOR OF RIPK3-DEPENDENT NECROPTOSIS

5.1 INTRODUCTION

Although necrosis generally results from severe pathological insults, recent studies suggest that certain membrane receptor ligands, such as TNF or Fas, can promote a form of programmed necrosis, also called necroptosis, in some cell types (Holler et al., 2000; Lin et al., 2004; Vandenabeele et al., 2010; Vercammen et al., 1998). TNF and Fas receptor stimulation can promote apoptosis via TRADD/FADD-dependent recruitment of caspase-8 to the intracellular death domains of these receptors (Ashkenazi and Dixit, 1998; Wang et al., 2008). However, TNF or Fas receptor engagement can result in necroptosis, in cells that express RIPK3, when receptor stimulation occurs in the presence of pharmacologic or viral-derived caspase inhibitors (Holler et al., 2000; Vercammen et al., 1998). In cells lacking RIPK3, inhibition of caspase activity completely blocks TNF-induced cell death, but in RIPK3-expressing cells results in a necrotic-like mode of cell death. Similarly, targeted disruption of the *caspase-8* or *FADD* loci leads to embryonic lethality due to ectopic necrosis early in development (Dillon et al., 2012; Kaiser et al., 2011; Oberst et al., 2011; Zhang et al., 2011). Thus, necroptosis represents an alternative mode of cell death that is triggered in RIPK3-expressing cells when caspase activation is interfered with. The re-directing of TNF- or Fas-initiated signals could be beneficial for certain pathogens, through terminating TNF-dependent pro-inflammatory responses for example. As demonstrated in chapter four, necroptosis potently attenuated secretion of numerous inflammatory mediators. Alternatively, TNF-induced necroptosis could act as a safeguard against pathogens that interfere with pro-inflammatory signaling, by killing cells harboring such pathogens (Kaiser et al., 2013; Sridharan and Upton, 2014).

At present, necroptosis is thought to result from TNFR-, TLR4- or TLR3-dependent activation of RIPK1, leading to phosphorylation, oligomerization and activation of RIPK3 (Vandenabeele et al., 2010). Deregulated RIPK3 activation leads to phosphorylation and oligomerization of MLKL (Cai et al., 2014; Sun et al., 2012) which triggers relocalization of the latter to the plasma membrane (Wang et al., 2014). Oligomerized MLKL promotes necroptosis by a mechanism that has yet to be fully resolved but may involve activation of TRPM7 calcium channels (Cai et al., 2014) or direct permeabilization of the plasma membrane by MLKL oligomers (Wang et al., 2014).

Although it is widely believed that RIPK1 is required for phosphorylation and activation of RIPK3 in the TNF pathway to necroptosis as well as other contexts (Cho et al., 2009; He et al., 2009), the role that RIPK1 plays in necroptosis is somewhat unclear. A key reason that RIPK1 kinase activity is thought to be required for necroptosis is that Necrostatin-1, a RIPK1 kinase inhibitor, potently inhibits this mode of cell death in numerous contexts (Degterev et al., 2008; Duprez et al., 2011; Zheng et al., 2008). Indeed, Necrostatin-1-inhibitable cell death has practically become a defining characteristic of necroptosis. Furthermore, because necroptosis appears to occur under certain pathological conditions *in vivo*, Necrostatin-1 analogues are currently undergoing evaluation as possible therapeutic agents (Degterev et al., 2005; Linkermann and Green, 2014). However, inhibition of necroptosis by RIPK1 inhibitors may not necessarily reflect a requirement for RIPK1 kinase activity for this mode of cell death to proceed. It is also possible that inhibition of RIPK1 kinase activity could alter the function of this molecule to generate a dominant-negative inhibitor of necroptosis.

There are a number of observations at odds with the view that RIPK1 is a positive regulator of necroptosis. Firstly our own observations; in chapter four, we demonstrated that necroptosis attenuates TNF-induced inflammation. During the study, we wanted to ensure the death we were observing with TNF/zVAD treatment was indeed necroptosis. As necroptosis is defined as a RIPK1 and RIPK3 dependant necrosis, we knocked down RIPK1 and RIPK3 expecting to find that knock down of either would block cell death. Indeed, RIPK3 knockdown blocked TNF/zVAD-induced necroptosis of L929 cells and MEFS. However, in Figure 4.5, RIPK1 knockdown clearly potentiated necroptosis while Necrostatin-1 blocked it. We found this observation intriguing so we decided to investigate further. Indeed, several reports suggest that RIPK1 is dispensible for necroptosis in some settings (Moujalled et al., 2013; Upton et al., 2012; Vanlangenakker et al., 2011). Second, whereas *RIPK3*$^{-/-}$ mice are viable (Newton et al., 2004), targeted inactivation of *RIPK1* is lethal, possibly due to enhanced apoptosis as a consequence of reduced RIPK1-dependent NFκB activation (Kelliher et al., 1998; Zhang et al., 2011). However, targeted inactivation of *caspase-8*, which should suppress ectopic apoptosis generated through loss of *RIPK1*, fails to rescue the *RIPK1* knockout

phenotype (Dillon et al., 2014; Rickard et al., 2014). The latter observation is at odds with the idea that *RIPK1*$^{-/-}$ animals die as a result of excessive apoptosis. Furthermore, deletion of *RIPK3* also fails to suppress lethality due to *RIPK1* loss (Dillon et al., 2014; Kaiser et al., 2014; Rickard et al., 2014), whereas *RIPK3* loss does suppress lethality due to *caspase-8* or *FADD* deletion, suggesting that the latter animals die due to excessive necroptosis (Dillon et al., 2012; Oberst et al., 2011). Rather surprisingly, three recent reports suggest that combined loss of *RIPK3* and *caspase-8* blocks the lethal effect of *RIPK1* deletion (Dillon et al., 2014; Kaiser et al., 2014; Rickard et al., 2014). These observations suggest that RIPK1 acts simultaneously to repress Caspase-8-dependent apoptosis, as well as RIPK3-dependant necroptosis. Therefore, rescue of *RIPK1* null animals requires the elimination of both *caspase-8* (to block apoptosis) as well as *RIPK3* (to block necroptosis). These data suggest that the role of RIPK1 as a positive regulator of necroptosis requires further clarification.

Here we have explored the requirement of RIPK1 for TNF-, LPS- and polyIC-initiated necroptosis and demonstrate that not only is RIPK1 dispensible for this process, but that knockdown of RIPK1 led to greatly accelerated necroptosis. Moreover, while the RIPK1 kinase inhibitor Necrostatin-1 blocked necroptosis in the presence of RIPK1, it failed to do so when RIPK1 expression was ablated. Collectively, these data suggest that RIPK1 participates in necroptosis as an inhibitor of this process, a function that is strongly enhanced via Necrostatin-1, most likely through converting RIPK1 into a dominant-negative inhibitor of RIPK3 activation.

5.2 RESULTS

5.2.1 TNF induces RIPK3- and MLKL-dependent necroptosis

As previous studies have shown, TNFR stimulation in RIPK3-expressing L929 cells can be switched to necroptosis through inhibition of caspase activity (Vercammen et al., 1998). As can be seen from (Figure 5.1 A and B), the poly-caspase inhibitor zVAD-fmk resulted in TNF-induced necroptosis of L929 cells within 5-6 h of receptor stimulation. TNF/zVAD-induced necroptosis was strongly suppressed by

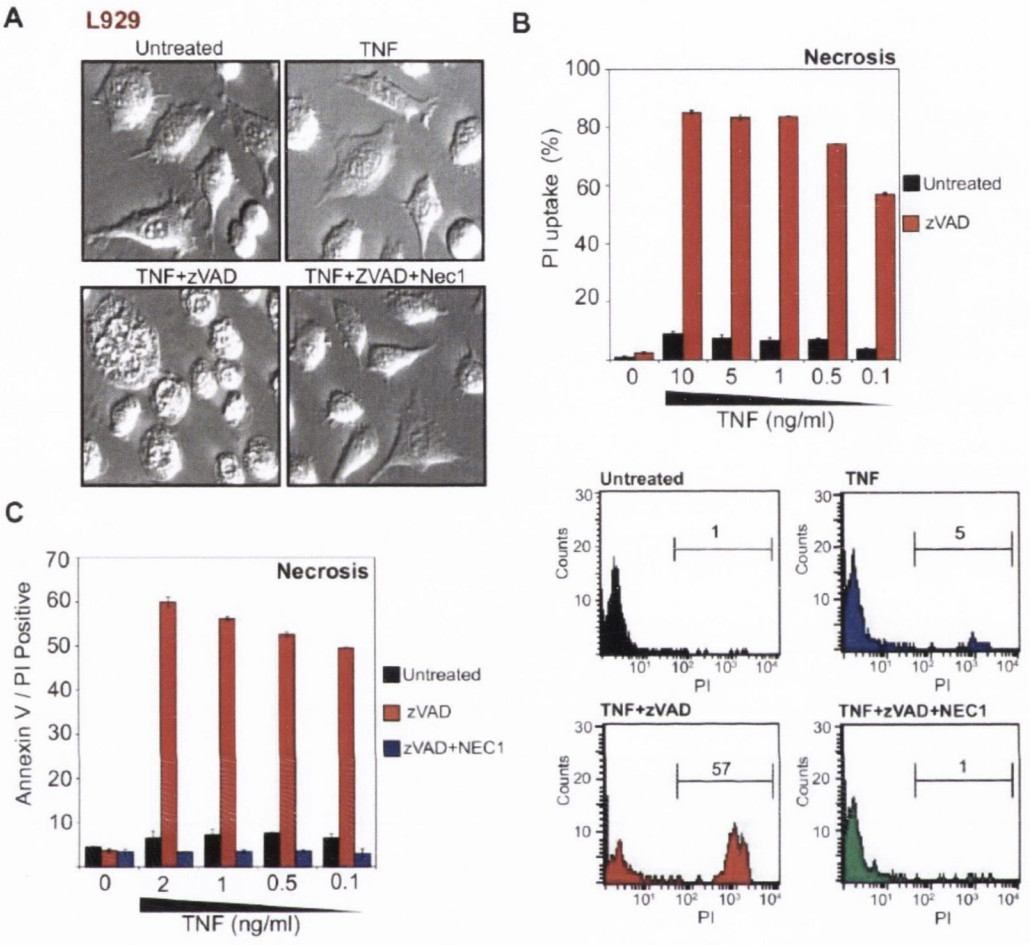

Figure 5.1

TNF induces necroptosis of L929 cells that is accelerated by zVAD. (A) L929 cells were treated with TNF (10 ng/ml), TNF/zVAD (10 μM) or TNF/zVAD/necrostatin (10 μM) for 6 hours then viewed by phase contrast microscopy. **(B)** L929 cells were left untreated, or treated with zVAD (10 μM) for 1 h, followed by addition of TNF to the indicated concentrations. After 6 hours, cell death was analyzed by propidium iodide staining. **(C)** L929 cells were left untreated, or treated with zVAD (10 μM) in the presence or absence of necrostatin-1 (10 μM), followed by addition of TNF to the indicated concentrations. After 6 hours, cell death was analyzed by propidium iodide staining. Representative flow cytometry charts are shown on the right.

the RIPK1 inhibitor, Necrostatin-1, suggesting that the kinase activity of RIPK1 is required for necroptosis (Figure 5.1 A and C). Furthermore, knockdown of RIPK3 or MLKL also readily protected from TNF-induced necroptosis (Figure 5.2). Thus, upon inhibition of caspase activity, TNF-induces necroptosis in a RIPK3- and MLKL-dependent manner as recently reported (Sun et al., 2012).

5.2.2 Silencing of RIPK1 expression accelerates TNF-induced necroptosis

Because Necrostatin-1 is a RIPK1 kinase inhibitor (Degterev et al., 2008; Xie et al., 2013) it is widely believed that RIPK1 kinase activity is required to promote RIPK3 activation and necroptosis (Cho et al., 2009; Ofengeim and Yuan, 2013; Vandenabeele et al., 2010). However, an alternative interpretation of the effects of Necrostatin-1 is that the kinase-inhibited form of RIPK1 could act as a dominant-negative inhibitor of necroptosis. To clarify the requirement of RIPK1 for TNF-induced necroptosis, we silenced the expression of this kinase and explored the impact of this on TNF/zVAD-induced necroptosis in L929 cells. However, knockdown of RIPK1 not only failed to protect from TNF-induced necroptosis, ablation of RIPK1 expression clearly potentiated this process (Figure 5.3 A and B). In contrast, knockdown of RIPK3 robustly protected from TNF-induced necroptosis (Figure 5.3 A and B). Potentiation of TNF-induced necroptosis as a consequence of RIPK1 knockdown was observed over a wide range of TNF concentrations (Figure 5.4). Thus, in opposition to the idea that RIPK1 kinase activity is required for necroptosis, these data suggest that RIPK1 acts as an inhibitor of this process in certain contexts.

To exclude the possibility that knockdown of RIPK1 enhanced TNF/zVAD-induced cell death in a manner unrelated to the necroptotic pathway, we also asked whether the enhanced necroptosis seen upon knockdown of RIPK1 was RIPK3-dependent. However, sensitization to TNF/zVAD-induced necrosis by knockdown of RIPK1 was completely abolished through simultaneous knockdown of RIPK3, arguing that death in this context was necroptotic (Figure 5.3). Several studies have shown that RIPK3 forms oligomers during necroptotic conditions and this can be detected as

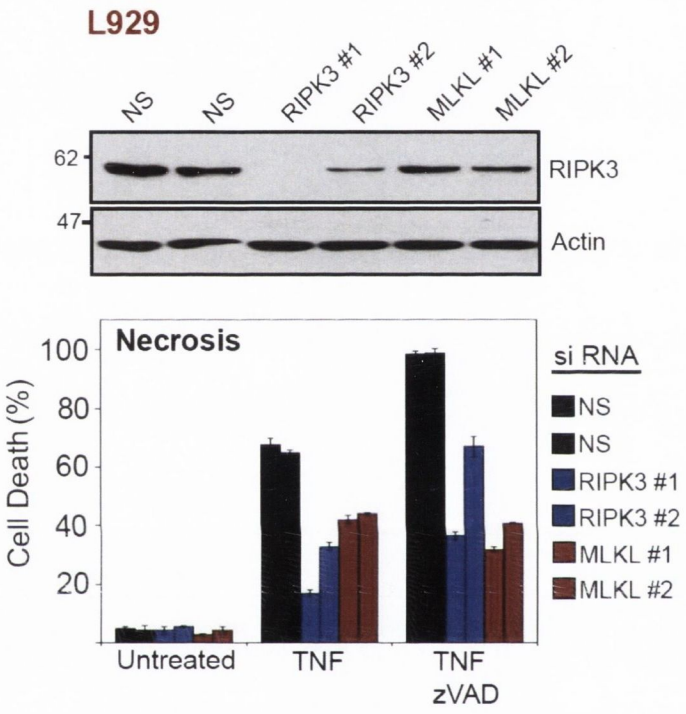

Figure 5.2

TNF induced necroptosis is RIPK3 and MLKL-dependent. L929 cells were electroporated with the indicated siRNAs. After 48 hours, cells were either left untreated, or were treated with zVAD (10 μM) for 1 h, followed by addition of TNF to 10 ng/ml as indicated. After a further 24 h, cell death was analyzed by morphological assessment of a minimum of 300 cells per treatment. RIPK3 knockdown was assessed by immunoblotting, upper panels.

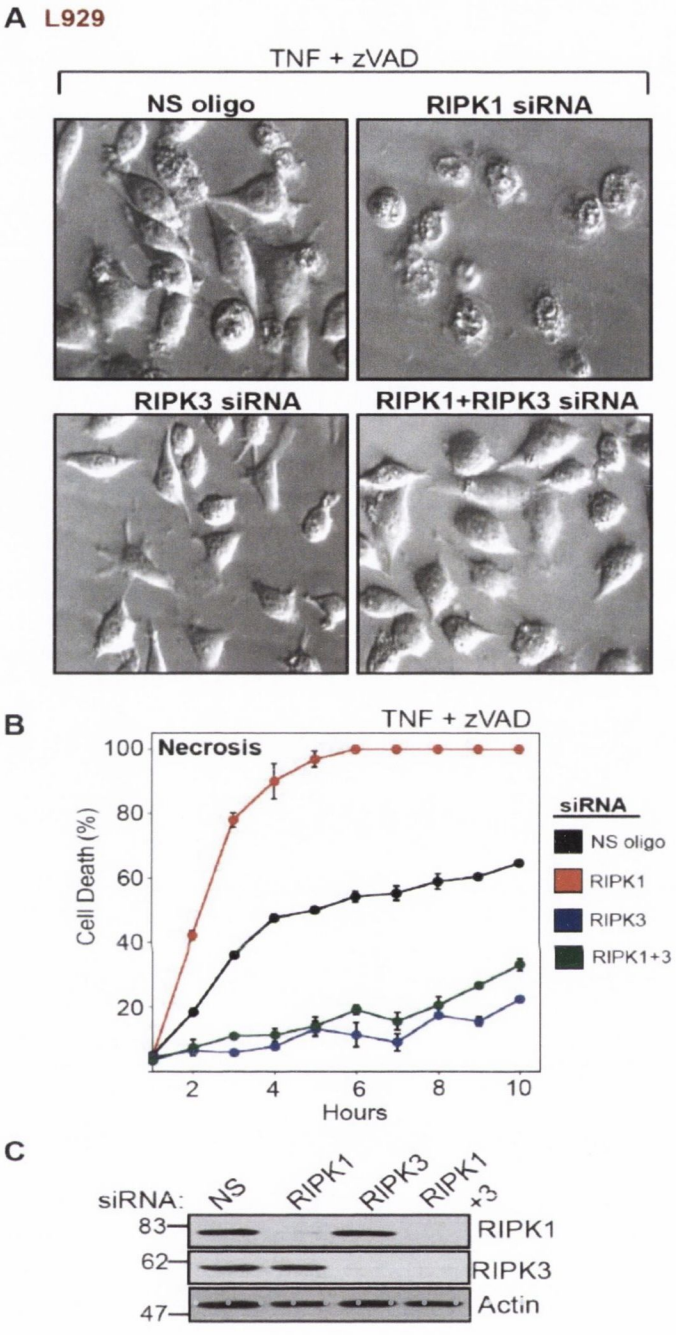

Figure 5.3

RIPK1 knockdown accelerates TNF-induced necroptosis. (A) L929 cells were electroporated with the indicated siRNAs. After 48 h, cells were treated with zVAD (5 μM) for 1 h, followed by addition of TNF to 200 pg/ml. After 6 hours, cells were viewed by phase contrast microscopy. **(B)** L929 cells were electroporated and treated as in *A*, then cell death analysed at the indicated time points by morphological assessment. **(C)** RIPK1 and RIPK3 expression were determined by immunoblotting 48 h after the siRNA electroporation described in *A*.

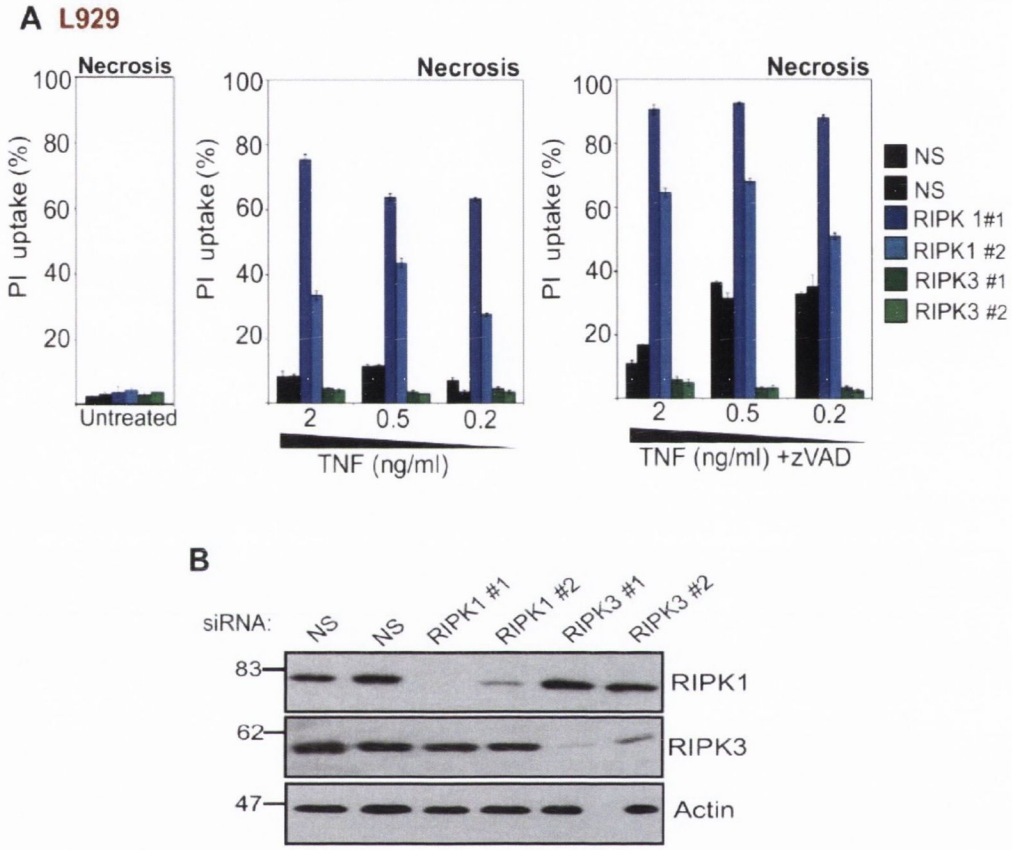

Figure 5.4

RIPK1 knockdown accelerates necroptosis over a concentration range of TNF. (A) L929 cells were electroporated with the indicated siRNAs. After 48 h, cells were either left untreated or treated with zVAD (5 μM) for 1 h, followed by addition of TNF to the indicated concentrations. After a further 6 h, cell death was analysed by propidium iodide staining. **(B)** RIPK1 and RIPK3 expression levels were determined by immunoblotting 48 h after electroporation

higher molecular weight forms on non-denaturing SDS-PAGE. As demonstrated in Figure 5.5, we also confirmed that the formation of RIPK3 oligomers in response to TNF/zVAD-treatment was enhanced through knockdown of RIPK1.

To explore whether TNF-induced necroptosis was also RIPK1 independent in another cell type we used primary MEFs, which also underwent necroptosis in response to TNF/zVAD treatment (Figure 5.6 A and B). TNF/zVAD-induced necrosis in MEFs was also inhibited by Necrostatin-1, again suggesting a requirement for RIPK1 kinase activity (Figure 5.6 C). However, as observed in L929 cells, knockdown of RIPK1 in MEFs failed to protect from TNF/zVAD-induced necroptosis, and again resulted in higher levels of necroptosis in response to TNF/zVAD-treatment (Figure 5.7). Once again, TNF/zVAD-induced necroptosis was robustly inhibited via RIPK3 knockdown in the same context (Figure 5.7 A and B), and the enhanced necroptosis seen as a consequence of RIPK1 knockdown was blocked by concurrent ablation of RIPK3 (Figure 5.7 A and B).

These observations suggest that RIPK1 is a negative rather than a positive regulator of necroptosis, whereas RIPK3 is indispensable for this process.

5.2.3 RIPK1 is dispensable for LPS and poly IC-induced necroptosis

Because necroptosis can also be invoked in response to LPS/TLR4 stimulation in the presence of caspase inhibitors, we next explored whether this form of necroptosis also required RIPK1 or RIPK3. As shown in Figure 5.8, LPS induced robust necroptosis in RAW macrophages in the presence of zVAD-fmk. LPS-induced necroptosis was efficiently blocked by necrostatin, as expected (Figure 5.8). However, silencing of RIPK1 expression failed to block LPS/zVAD-induced necroptosis and again resulted in greatly accelerated necroptosis, whereas knockdown of RIPK3 was protective (Figure 5.9 and 5.10 A). Again, we observed increased formation of RIPK3 oligomers in the absence of RIPK1 following LPS/zVAD treatment (Figure 5.10 B). As we observed in the context of TNF-

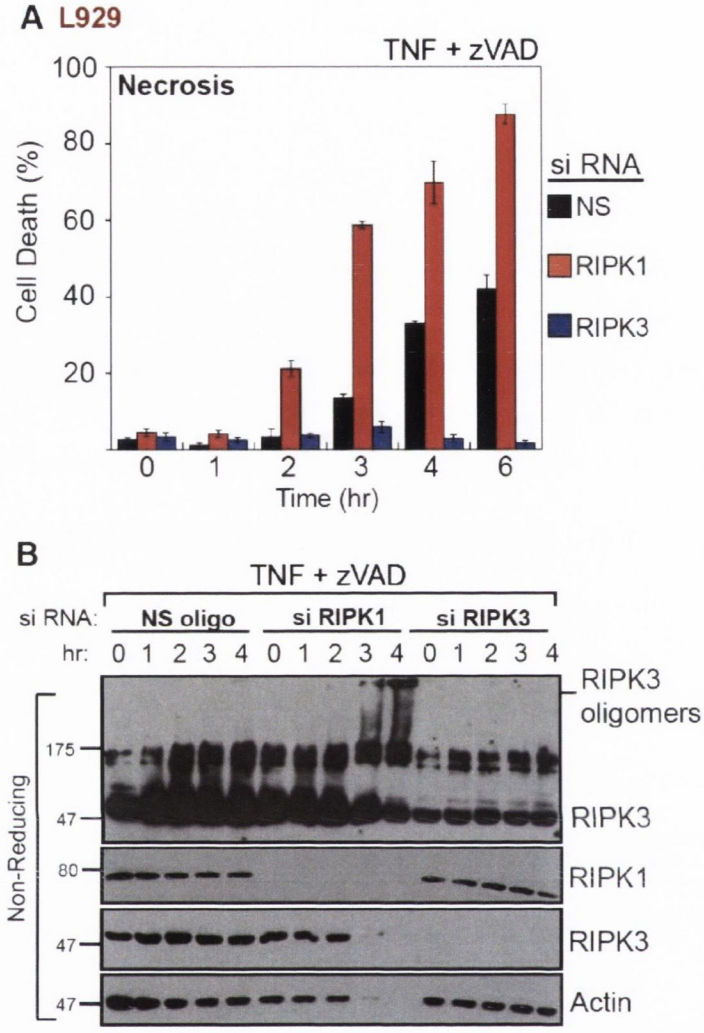

Figure 5.5

RIPK1 knockdown accelerates TNF-induced formation of RIPK3 oligomers. **(A)** L929 cells were electroporated with the indicated siRNAs. After 48 h, cells were treated with zVAD (10 μM) for 1 h, followed by addition of TNF to 500 pg/ml. At the indicated time points, cell death was analyzed by morphological assessment. A minimum of 300 cells were counted per treatment. **(B)** At the indicated time points, the cell cultures from *A* were analysed for the formation of RIPK3 oligomers, by non-reducing SDS-PAGE

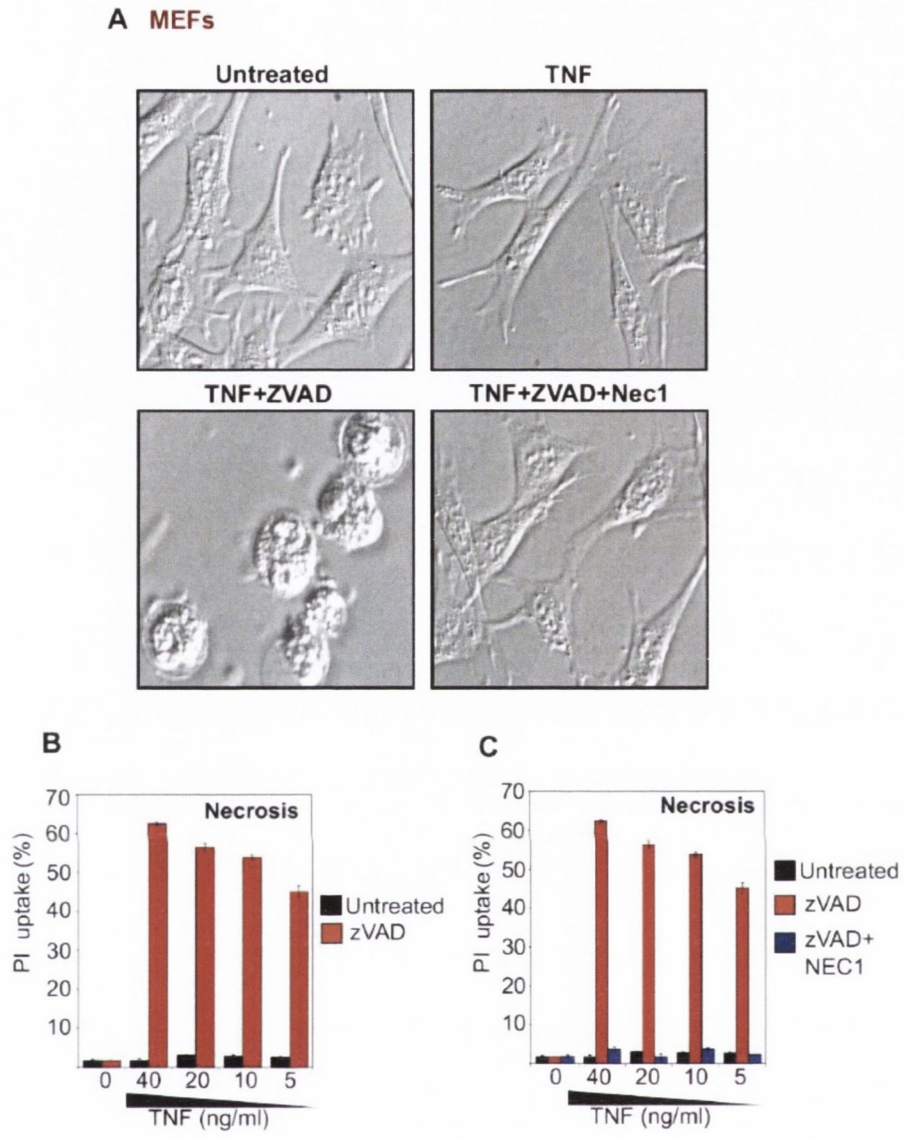

Figure 5.6

MEFs undergo TNF-induced necroptosis in the presence of zVAD. (A) MEFs were left untreated, or treated with zVAD (20 μM) in the presence or absence of necrostatin-1 (5 μM) for 1 h, followed by addition of TNF to 40 ng/ml. After a further 6 h, cells were viewed by phase contrast microscopy. **(B)** MEFs were left untreated, or treated with zVAD (20 μM) in the presence or absence of necrostatin-1 (5 μM) **(C)**, followed by addition of TNF to the indicated concentrations. After 24 h, cell death was analyzed by propidium iodide staining.

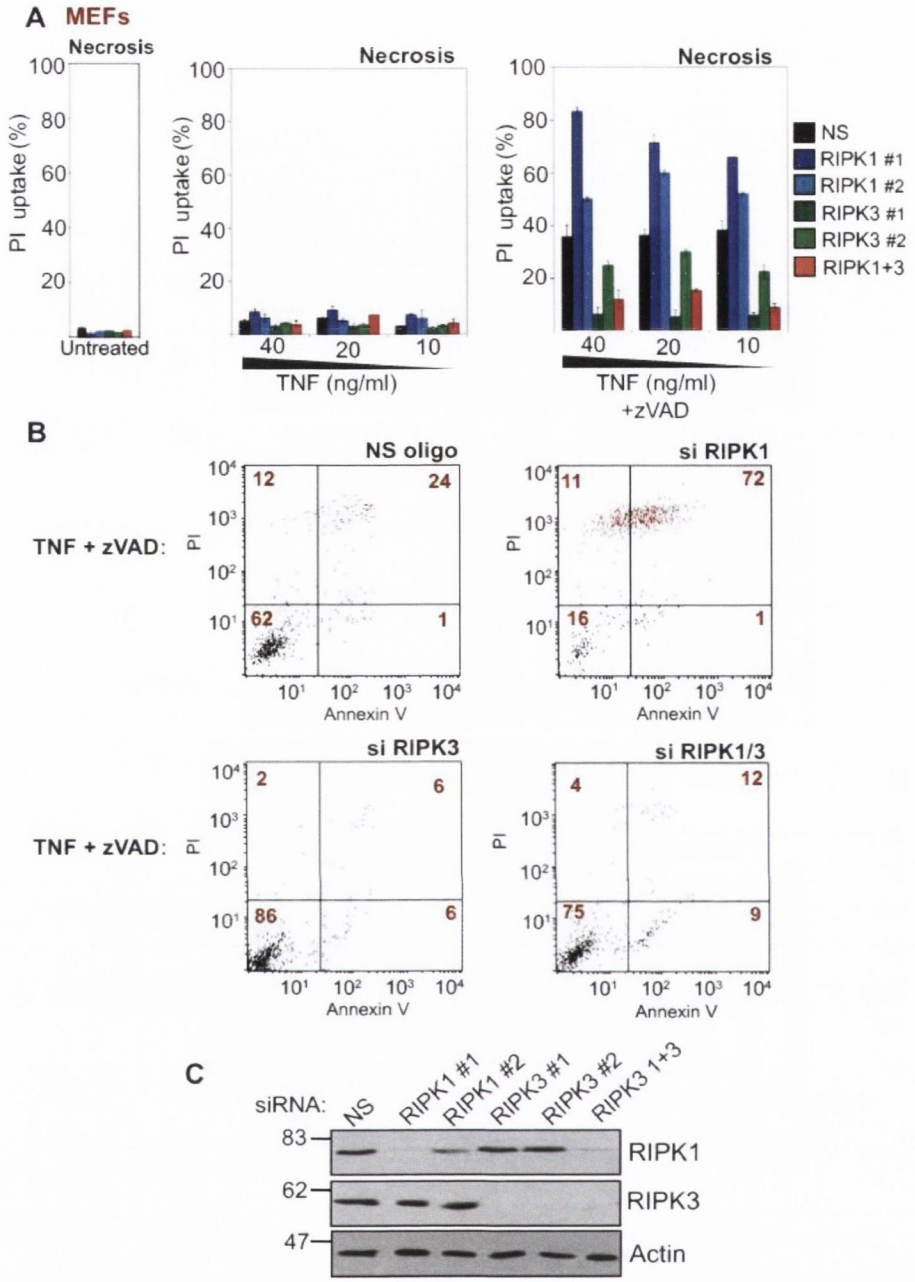

Figure 5.7

RIPK1 knockdown potentiates TNF-induced necroptosis of MEFs. (A) MEFs were electroporated with the indicated siRNA. After 48 h, cells were either left untreated, or treated with zVAD (10 μM) for 1 h, followed by addition of TNF to the indicated concentrations. After a further 6 h, cell death was analyzed by propidium iodide staining. **(B)** Representative flow cytometry charts of cells treated with the siRNA as in *A*, followed by addition of TNF/zVAD for 6 h. **(C)** RIPK1 and RIPK3 expression levels from *A* and *B*, were determined by immunoblotting.

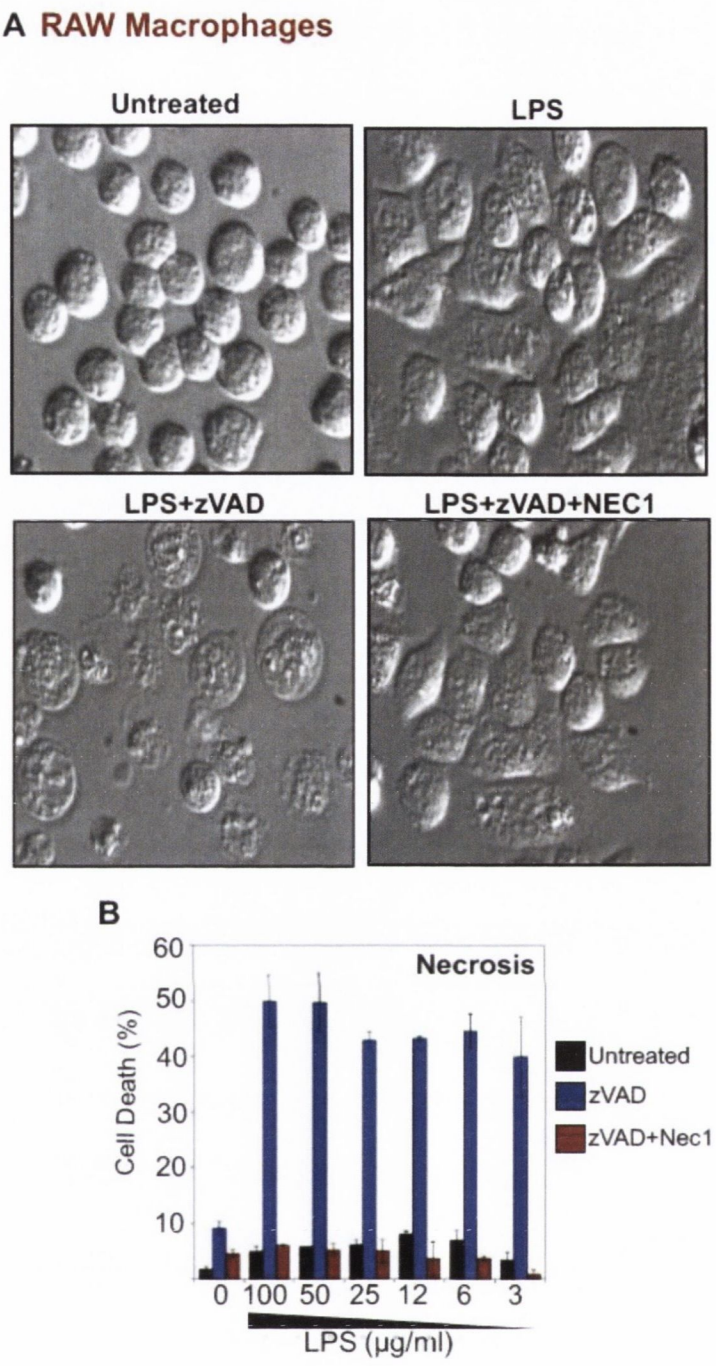

Figure 5.8

LPS induces necroptosis of RAW macrophages in the presence of zVAD. (A) RAW cells were treated left untreated or treated with zVAD (10 μM) in the presence or absence of necrostatin-1 (5 μM) for 1 h, followed by addition of LPS to 50 μg/ml. After 24 h, cells were viewed by phase contrast miscroscopy. **(B)** RAW cells were treated left untreated or treated with zVAD (10 μM) in the presence or absence of necrostatin-1 (5 μM) for 1 h, followed by addition of LPS to the indicated concentrations. After 24 h, cell death was analysed by morphological assessment. A minimum of 300 cells were counted per treatment.

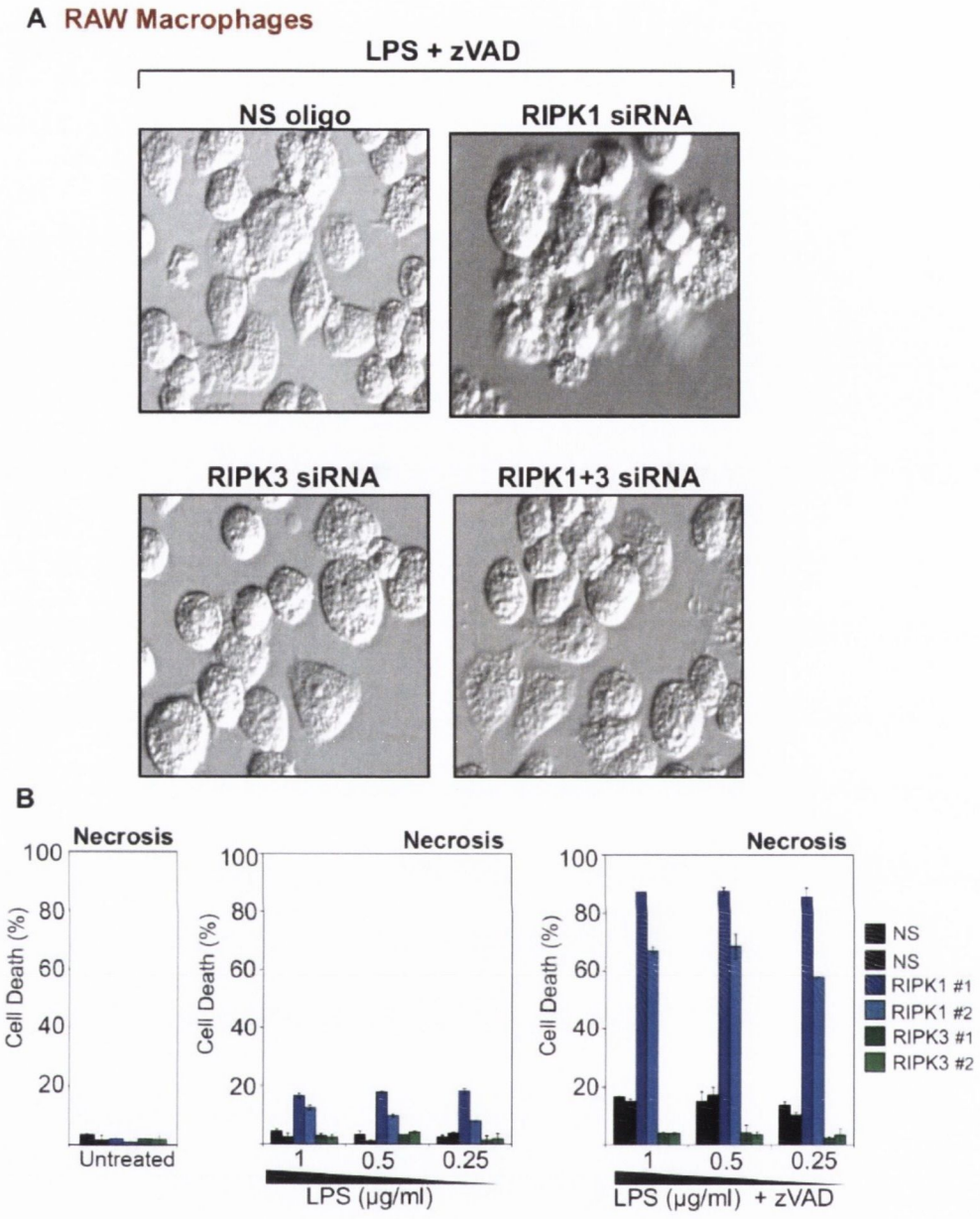

Figure 5.9

RIPK1 suppresses LPS-induced necroptosis. (A) RAWs were electroporated with the indicated siRNA. After 48 h, cells were treated with zVAD (10 μM) for 1 h, followed by addition of LPS to 1 μg/ml. After 24 h, cells were visualized by phase contrast microscopy. **(B)** RAWs were electroporated with the indicated siRNAs. After 48 h, cells were either left untreated, or treated with zVAD (10 μM) for 1 h, followed by addition of LPS to the indicated concentrations. After a further 24 h, cell death was analyzed by morphological assessment. A minimum of 300 cells were counted per treatment.

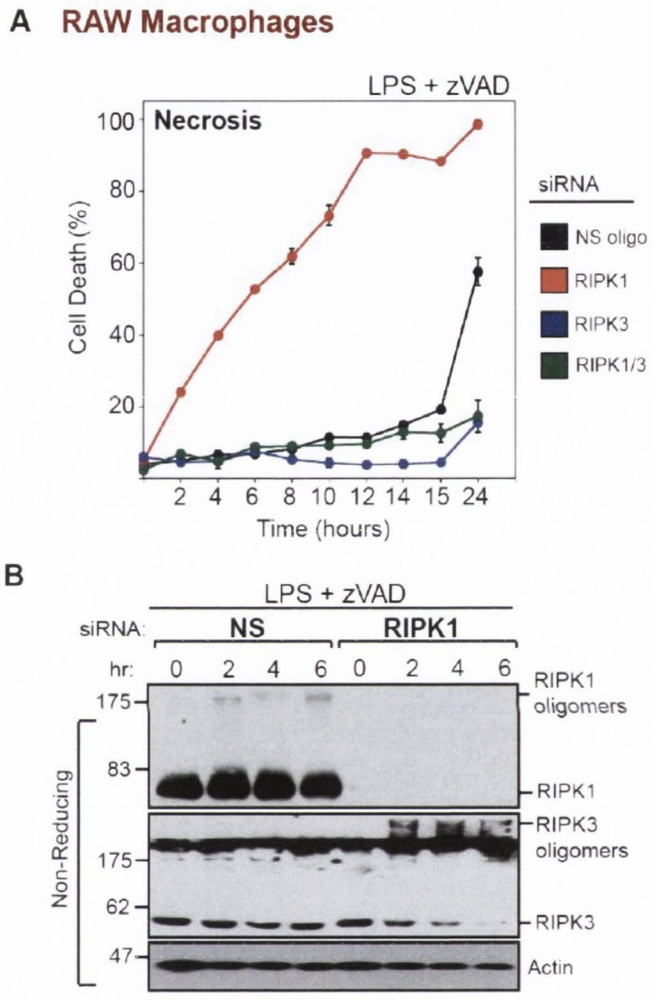

Figure 5.10

RIPK1 suppresses LPS / zVAD-induced formation of RIPK3 oligomers. (A) RAWs were electroporated with the indicated siRNAs. After 48 h, cells were treated with zVAD (10 μM) for 1 h, followed by addition of LPS to 1 μg/ml. At the indicated time points, cell death was analyzed by morphological assessment. A minimum of 300 cells were counted per treatment. **(B)** At the indicated time points, cell cultures from *A* were analysed for the formation of RIPK1 and RIPK3 oligomers by non-reducing SDS-PAGE.

induced necroptosis, the enhanced necroptosis seen due to knockdown of RIPK1 was completely abolished through concurrent ablation of RIPK3 (Figure 5.10 B).

Treatment with the TLR3 ligand, polyIC, also promotes necroptosis (He et al., 2011). Thus we also explored whether RIPK1 was dispensable for necroptosis in this setting. We found that RAW macrophages were relatively resistant to PolyIC-induced necroptosis in the presence of endogenous RIPK1, even at high concentrations of polyIC (Figure 5.11 A). However, knockdown of RIPK1 greatly sensitized towards polyIC-induced necroptosis in RAW cells, whereas knockdown of RIPK3 did not (Figure 5.11 A). Once again, the increased sensitivity to polyIC-initiated necroptosis seen upon knockdown of RIPK1 was abolished through simultaneous knockdown of RIPK3 (Figure 5.11 A). Sensitization to PolyIC- induced necroptosis, as a consequence of RIPK1 ablation was displayed typical necrotic phenotypic characteristics (Figure 5.12 A) and was observed over a range of polyIC concentrations (Figure 5.12 B).

5.2.4 Necrostatin-1 blocks necroptosis only in the presence of RIPK1

Because ablation of RIPK1 expression failed to block necroptosis in response to TNF, LPS or polyIC treatment, but the RIPK1 inhibitor Necrostatin-1 did so, this suggested either that Necrostatin-1 was operating in an off-target manner, or that the role of RIPK1 in necroptosis is more complex than currently understood. To resolve whether Necrostatin-1 has an off-target effect on necroptosis, we asked whether this inhibitor still protected from necroptosis upon knockdown of RIPK1. However, whereas Necrostatin-1 efficiently blocked TNF/zVAD -induced necroptosis in L929 cells in the presence of RIPK1 (Figure 5.13 A) it failed to do so upon knockdown of this kinase (Figure 5.13 B and C). Indeed, as demonstrated earlier, knockdown of RIPK1 greatly sensitized to necroptosis, which failed to be reversed by Necrostatin-1 (Figure 5.13 B and C; Figure 5.14). However, knockdown of RIPK3 concurrently with RIPK1 did protect from TNF/zVAD-induced necroptosis (Figure 5.13 B and C; Figure 5.14).

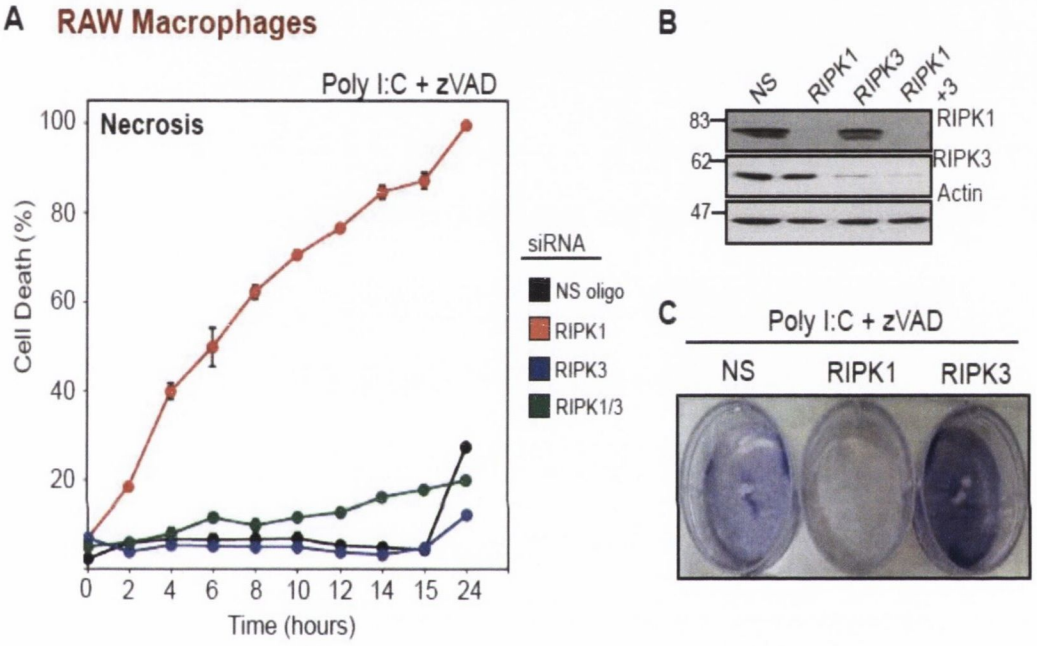

Figure 5.11

RIPK1 protects from TLR-3-induced necroptosis. (A) RAW cells were electroporated with the indicated siRNAs. After 48 h, cells were treated with zVAD (10 μM) for 1 h, followed by addition of polyIC to 10 μg/ml. At the indicated time points, cell death was analyzed by morphological assessment. A minimum of 300 cells were counted per treatment. **(B)** After 48 h following electroporation with the indicated siRNAs, RIPK1 and RIPK3 expression levels were determined by immunoblotting in the cell cultures from *A*. **(C)** RAW cells were electroporated with the indicated siRNAs. After 48 h, cells were treated with zVAD (10 μM) for 1 h, followed by addition of polyIC to 10 μg/ml. After 24 h, cell survival was examined by crystal violet staining.

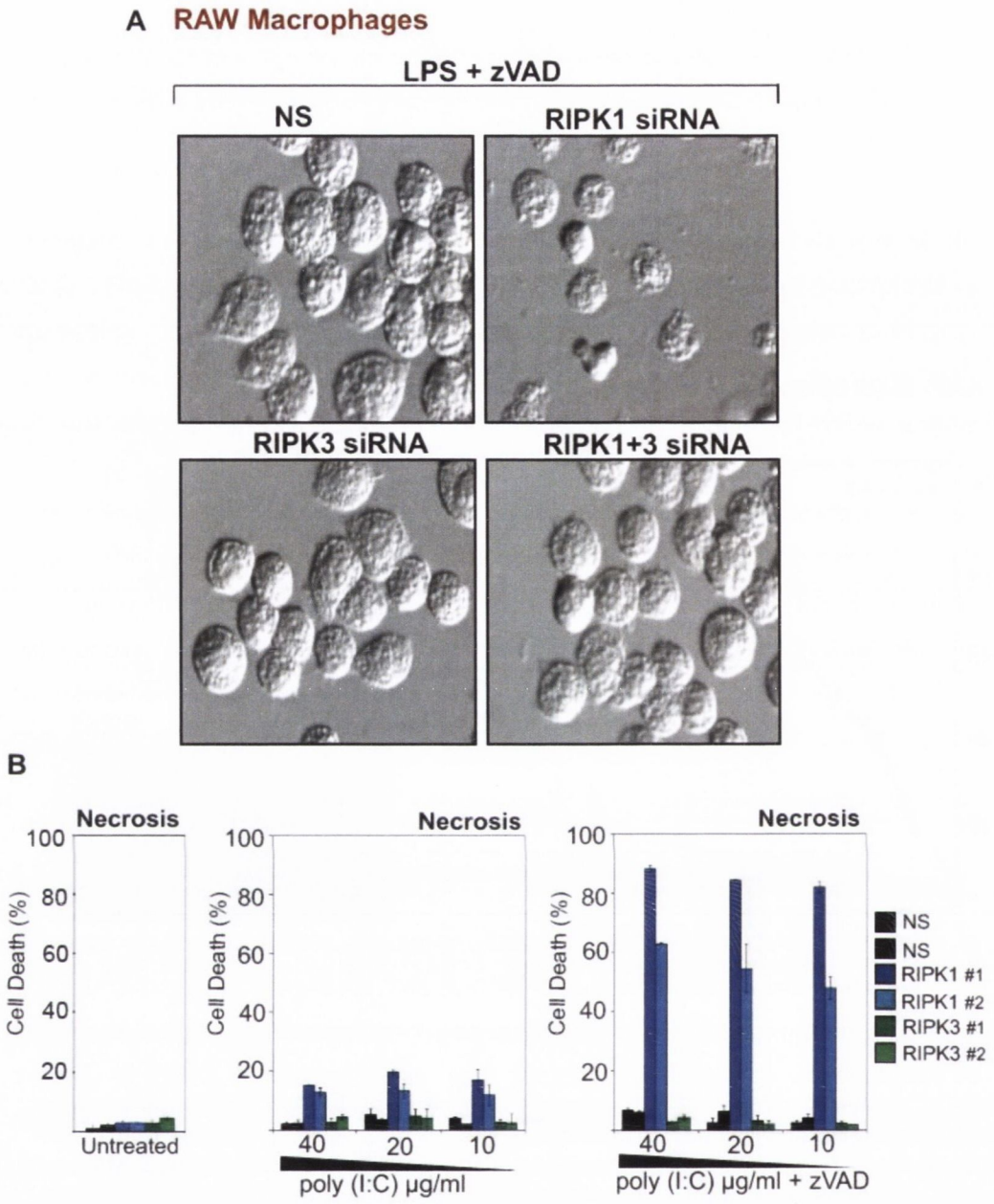

Figure 5.12

RIPK1 knockdown accelerates necroptosis over a concentration range of polyIC. (A) RAWs were electroporated with the indicated siRNAs. After 48 h, cells were treated with zVAD (10 µM) for 1 h, followed by addition of polyIC to 1 µg/ml. After 24 h, cells were visualized by phase contrast microscopy. **(B)** RAWs were electroporated with the indicated siRNAs. After 48 h, cells were either left untreated, or treated with zVAD (10 µM) for 1 h, followed by addition of polyIC to the indicated concentrations. After a further 24 h, cell death was analyzed by morphological assessment. A minimum of 300 cells were counted per treatment.

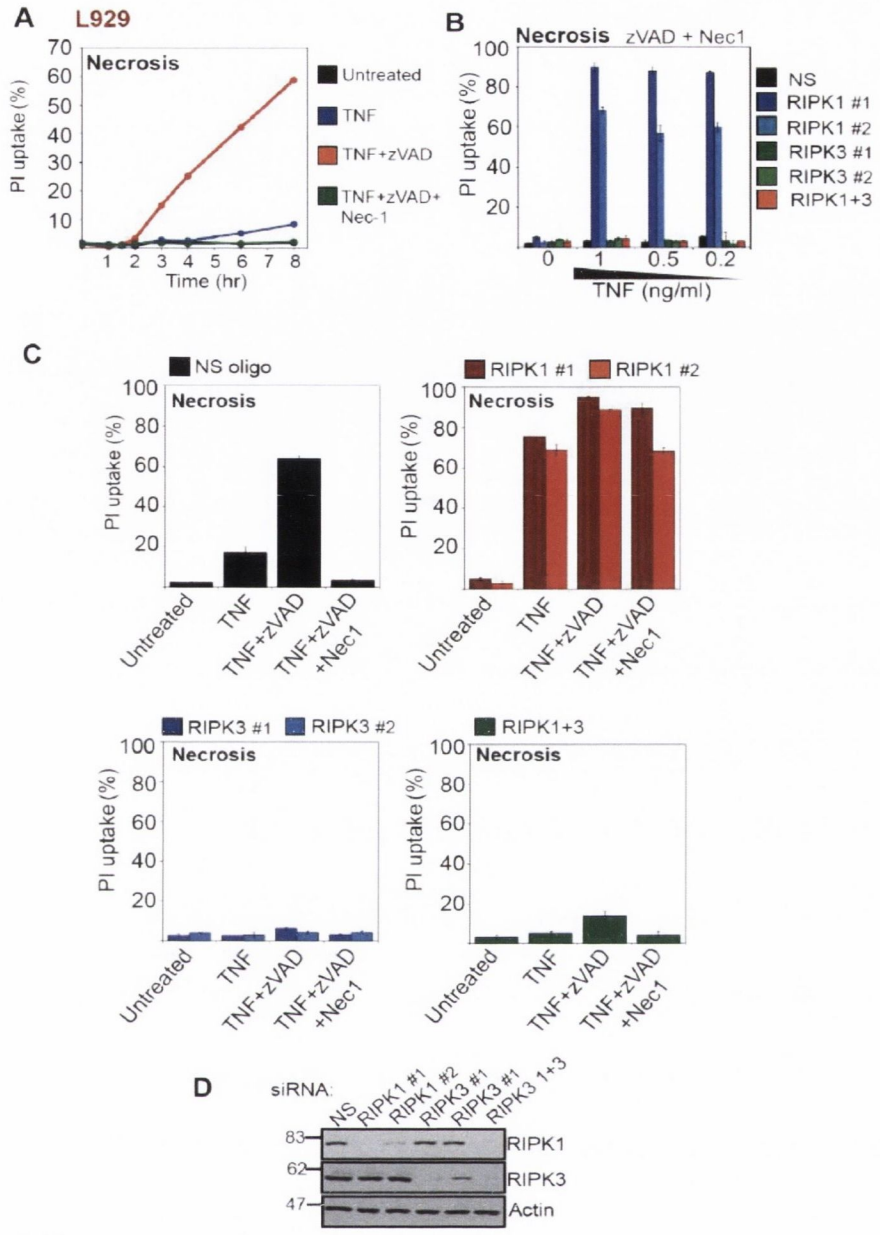

Figure 5.13

Necrostatin-1 fails to inhibit necroptosis in the absence of RIPK1. (A) L929 cells elecroporated with a non-silencing siRNA (NS oligo), or siRNA directed against RIPK1. After 48 h, cells were treated with zVAD (10 μM) in the presence or absence of necrostatin (5 μM) for 1 h, followed by addition of TNF to 10 ng/ml. After a further 8 h, cell death was analysed by ts, cell death was analyzed by morphological assessment. . A minimum of 300 cells were counted per treatment. **(B,C)** L929 cells were electroporated with the indicated siRNAs. After 48 h, cells were either left untreated, or treated with zVAD (10 μM) with or without necrostatin (5 μM) for 1 h, followed by addition of TNF to 1 ng/ml, or as indicated. After a further 8 h, Cell death was analyzed by propidium iodide staining. **(D)** Expression levels of RIPK1 and RIPK3 from *A* and *B*, was analysed by immunoblotting 48 h after electroporation.

RIPK1 associates with a necroptosis-inducing complex 'the necrosome' comprised of Caspase-8, RIPK1 and RIPK3, which is thought to serve as the MLKL activation platform (Vandenabeele et al., 2010). Necrostatin-1 is thought to prevent necroptosis by inhibiting RIPK1-mediated RIPK3 phosphorylation and oligomerization, which is required for MLKL activation downstream. We therefore asked whether RIPK1 was required for the ability of Necrostatin-1 to prevent RIPK3 oligomerization. As can be seen from Figure 5.15 A, silencing of RIPK1 accelerated cell death in the presence of Necrostain-1, in response to TNF/zVAD and correlated with the formation of RIPK3 oligomers (Figure 5.15 B). This demonstrates that Necrostatin-1 works by manipulating RIPK1 into a potent inhibitor of RIPK3 oligomerization, but in its native form, acts as a suppressor of RIPK3 oligomerization and subsequent necroptosis.

5.3 DISCUSSION

Here we have shown that RIPK1 is dispensable for necroptosis induced by TNF as well as by the TLR ligands LPS and polyIC. Furthermore, knockdown of RIPK1 greatly accelerated necroptosis induced by all of these stimuli, arguing that this kinase acts as an inhibitor of this process, a property that is potentiated by the RIPK1 inhibitor, Necrostatin-1. Consistent with this idea, whereas Necrostatin-1 efficiently blocked necroptosis when RIPK1 was present, it failed to have any effect when RIPK1 was absent. These data argue that Necrostatin-1 blocks necroptosis in a RIPK1-dependent manner, most likely through enhancing the inhibitory effects of RIPK1 on this mode of cell death. Importantly, the accelerated necroptosis seen upon knockdown of RIPK1 was fully RIPK3-dependent. Collectively, these data argue that RIPK1 can function as a suppressor of necroptosis and that RIPK1 kinase inhibitors block this mode of cell death in an unexpected way.

5.3.1 RIPK1 may protect from necroptosis by facilitating pro-survival gene induction

A major difference between inhibition of RIPK1 kinase activity using Necrostatin-1 and knockdown of RIPK1 is that Necrostatin-1 does not impede RIPK1-dependent

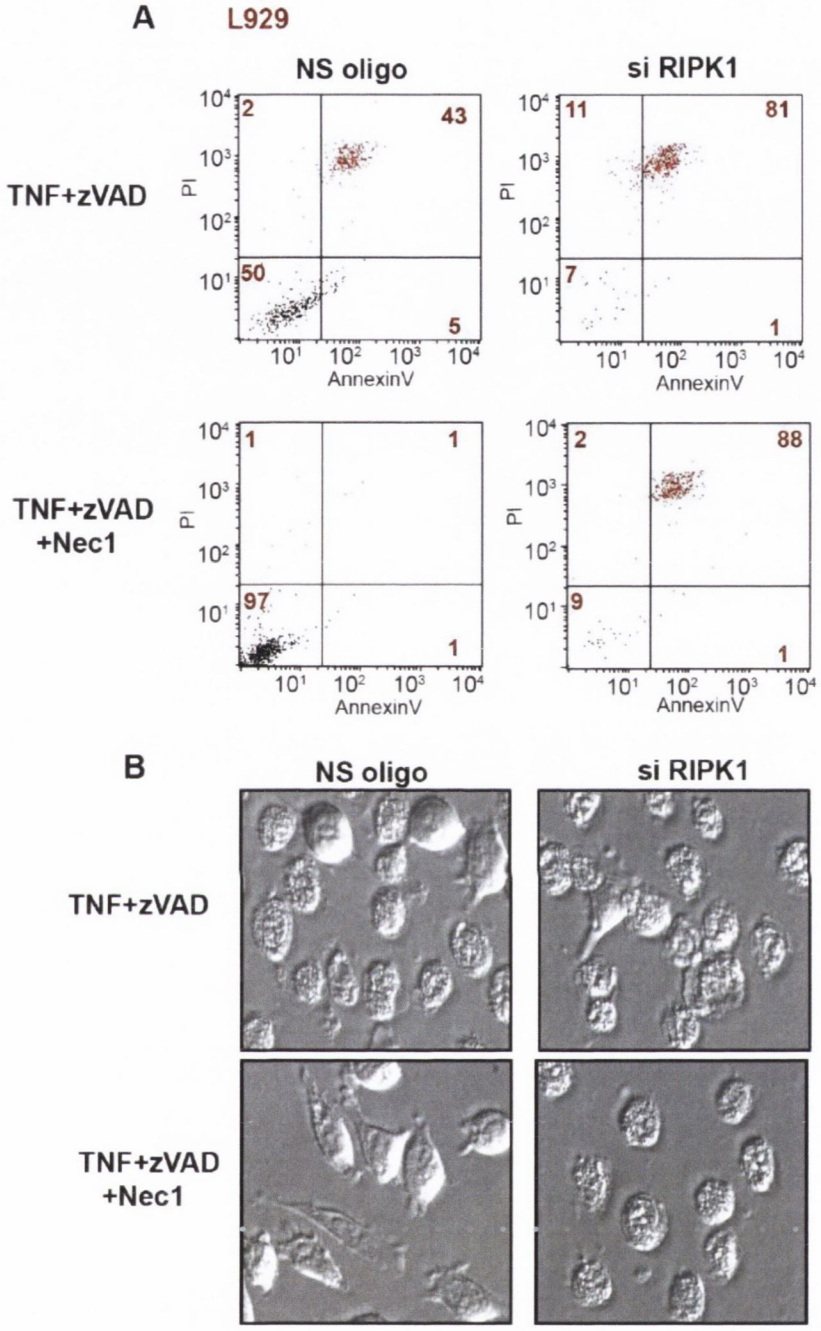

Figure 5.14

Necrostatin fails to inhibit necroptosis in the absence of RIPK1. (A) Representative flow cytometry plots from figure 5. Are displayed. **(B)** Representative phase contrast images from Figure 5. Are displayed.

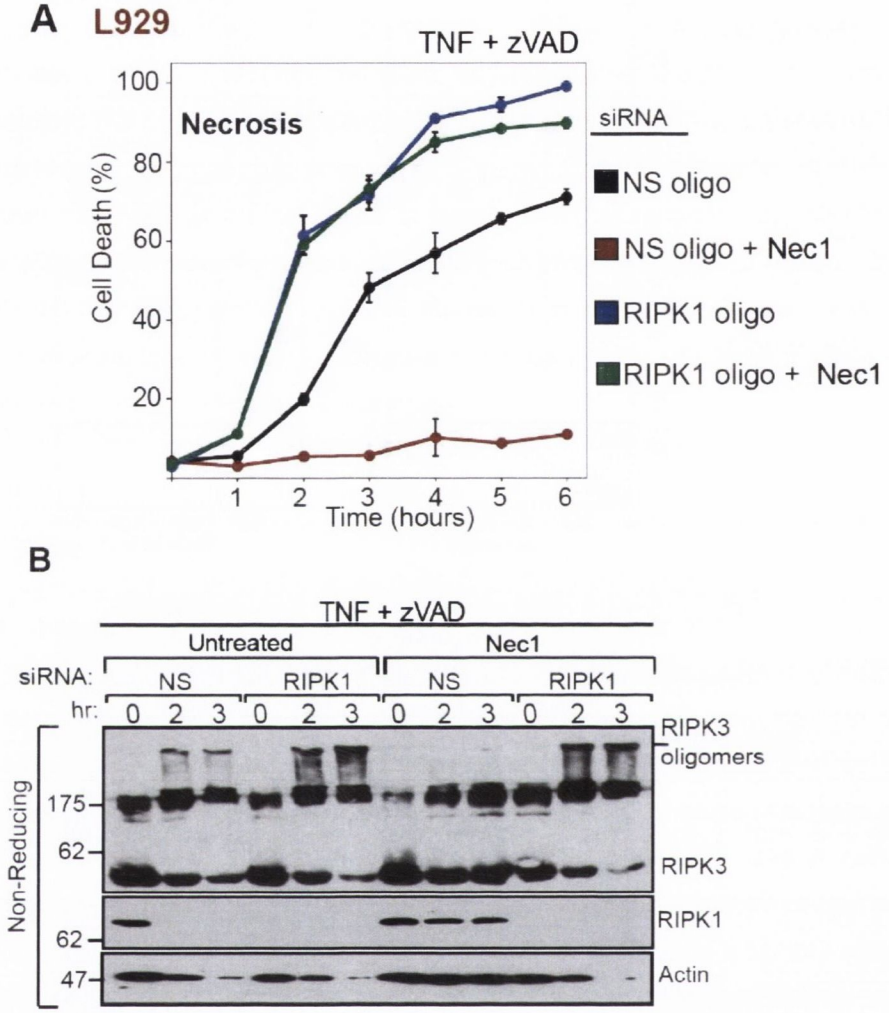

Figure 5.15

Necrostatin-1 blocks RIPK3 oligomerization, only in the presence of RIPK1. **(A)** L929 cells were electroporated with the indicated siRNAs. After 48 h, cells were treated with zVAD (10 μM) with or without necrostatin (5 μM) as indicated, for 1 h, followed by addition of TNF to 500 pg/ml. At the indicated time points, cell death was analyzed by morphological assessment. A minimum of 300 cells were counted per treatment. **(B)** At the indicated timepoints, cell cultures from *A*, were analysed for the formation of RIPK3 oligomers by non- reducing SDS-PAGE.

NFκB activation, as the kinase activity of the latter is not required for its role as a scaffold for IKKγ/NEMO recruitment and subsequent IKK2 activation. In contrast, knockdown of RIPK1 eliminates both its scaffold function in NFκB activation, as well as its kinase activity. It is possible that TNF-dependent up regulation of cIAPs or cFLIP is required to keep the threshold for necroptosis high, and RIPK1 may be required for optimal activation of pathways leading to transcription of these molecules. Alternatively, RIPK1 may be required to maintain steady state expression of death-protective molecules such as cFLIP and cIAPs. Therefore, knockdown of RIPK1 may lead to lower levels of these molecules, thus lowering the threshold for necroptosis. In support of this view, a recent study by Dannppel et al., demonstrated that cells derived from RIPK1 null mice had dramatically less expression levels of cFLIP and cIAP1 (Dannappel et al., 2014). The mechanism by which RIPK1 may maintain expression levels of such proteins, however, remains unresolved.

5.3.2 Genetically or chemically altered proteins can adopt drastically altered functions

Recently, Dixit and colleagues have demonstrated that knock-in of kinase dead RIPK3 results in embryonic lethality due to excessive yolk sack apoptosis (Newton et al., 2014). Unlike wild type RIPK3, the kinase dead RIPK3 mutant has the ability to associate with FADD and Caspase-8, thus driving excessive apoptosis. It is therefore possible that RIPK1, in its Necrostatin-1-bound kinase-inhibited form, is a potent inhibitor of necroptosis due to altered binding properties and not due to the loss of its kinase activity per se. Necrostatin-1 may promote a conformational change in RIPK1 that would now permit the Necrostatin-1-inhibited form of RIPK1 to act in a dominant-negative fashion, perhaps through binding and inhibiting RIPK3, or MLKL. Indeed Oberst and colleagues have shown that a kinase inactive RIPK1 mutant protected from necroptosis induced by a forced RIPK3 dimerizing construct (Orozco et al., 2014). However, this mutant is not as effective as Necrostatin-1 at blocking necroptosis. This suggests that particular conformational changes in RIPK1 create different inhibitory effects on the necroptosis pathway. It would be

interesting to identify a RIPK1 mutant that adopts a necroptosis inhibitory ability, as efficiently as Necrostatin-1.

5.3.3 Genetic evidence suggests RIPK1 prevents necroptosis and apoptosis

As mentioned earlier, very recent genetic studies also provide strong support for the idea that RIPK1 is a suppressor of RIPK3-dependent necroptosis, as well as Caspase-8-dependent apoptosis (Dillon et al., 2014; Kaiser et al., 2014; Rickard et al., 2014). Deletion of *RIPK1* is lethal at birth (Kelliher et al., 1998), but is only partly rescued by concurrent deletion of TNFR1 (Cusson et al., 2002). However, three recent studies have shown that lethality due to *RIPK1* loss is completely rescued through concurrent deletion of *caspase-8* and *RIPK3*, suggesting that *RIPK1*$^{-/-}$ animals dies due to excessive apoptosis as well as RIPK3-dependent necroptosis (Dillon et al., 2014; Rickard et al., 2014; Kaiser et al., 2014). Because TNFR1 deletion does not completely rescue *RIPK1* loss-mediated lethality, it suggests that TNF-independent cell death pathways are also initiated in the absence of RIPK1. Indeed, Dillon et al., have demonstrated that *RIPK1* loss-mediated lethality can be delayed by combined deletion of *TNFR1* with *TRIF* and *IFNAR*. This finding supports our data, suggesting that RIPK1 protects from interferon and toll ligand-induced necroptosis. The latter observations support the idea that RIPK1 is an important suppressor of RIPK3-dependent necroptosis *in vivo*.

The requirement for RIPK1 to suppress necroptosis *in vivo* seems to be dependent on tissue type. Conditional knockout of RIPK1 in the intestinal epithelium results in excessive inflammation and rapid lethality as a result of widespread apoptosis (Takahashi et al., 2014). Combined loss of caspase-8, but not RIPK3 rescued this phenotype, confirming that the cell death was indeed apoptosis, not necroptosis. In this model, kinase dead knock-in into the intestinal epithelium prevented apoptosis, demonstrating that it is the scaffold role of RIPK1 that prevents apoptosis, not the kinase function. Interestingly, cells derived from these RIPK1 depleted animals were not compromised in TNF-induced NFκB activation. This suggests that RIPK1

directly suppresses DISC formation in the intestine, independently of regulating transcription of pro-survival proteins such as cFLIP and IAPs. Therefore, particular tissues appear to have a higher propensity to activate RIPK3 in the absence of RIPK1, while other tissues revert to apoptotic cell death.

5.3.4 Do other RIPK3-activating kinases exist?

In the absence of RIPK1, it remains unclear whether RIPK3 undergoes autoactivation under necroptotic conditions or whether an upstream kinase is involved. We have found that RIPK3 oligomerizes more readily in the absence of RIPK1 suggesting that RIPK1 interferes with RIPK3 homo-interactions. Certainly, it appears that the kinase activity of RIPK1 is not responsible for triggering RIPK3 oligomerization, as this process occurs more rapidly in the absence of RIPK1. However, we do not know if an alternative kinase activates RIPK3 in the absence of RIPK1 or if RIPK3 simply oligomerizes more efficiently. Only four RHIM domain-containing proteins have been identified in the human genome to date; RIPK1, RIPK3, TRIF and DAI. The presence of a RHIM domain-containing adapter may be essential to facilitate recruitment of RIPK3 to the stimulated receptor. In the absence of RIPK1, it is possible that an as yet unidentified RHIM adaptor facilitates RIPK3 recruitment to the TNF receptor complex. Indeed, RIPK1 has been shown to be dispensable for necroptosis induced by LPS or viral detection but dependent on the RHIM adaptors TRIF and DAI respectively (He et al., 2011; Upton et al., 2012). However, in these settings it is not clear if RIPK3 interaction with TRIF or DAI is sufficient for RIPK3 activation, or if these RHIM-dependent interactions are recruiting RIPK3 into the proximity of an activating kinase.

In conclusion, we have shown that RIPK1 acts as an inhibitor of TNF- and TLR-induced necroptosis and that Necrostatin-1 blocks this mode of cell death in an unexpected way, most likely through enhancing the suppressive effects of RIPK1 on necroptosis.

CHAPTER 6

GENERAL DISCUSSION

6.1 INTRODUCTION

Throughout this thesis, TNF receptor stimulation and other innate immune receptors have been analyzed in the context of inflammation and cell death outcomes. While these two outcomes may initially appear separate and distinct processes, we have demonstrated that, in fact, it is important to study cell death and inflammation simultaneously.

In chapter one, three important questions were addressed; do IAPs influence TNF-driven inflammation independently of their role in cell death regulation? Does necroptosis diminish the inflammatory potential of the triggering ligand? Does RIPK1 function as a positive or negative regulator of necroptosis?

Members of the TNF superfamily, including TNF, can potently induce the production of pro-inflammatory cytokines and/or cell death, depending on cellular context. The inhibitor of apoptosis proteins and RIPK1 are critical in dictating whether TNF stimulation results in inflammation and/or cell death. Generally, it is believed that TNF receptor activation triggers recruitment of TRADD, RIPK1 and the IAPs. cIAP1 and cIAP2 then ubiquitinate RIPK1 to promote a signaling platform which leads to activation of multiple kinases. If RIPK1 fails to be ubiquitinated due to a block on NFkB activation or removal of IAPs, caspase-8 is activated, resulting in apoptosis. Importantly, we have shown that cIAP1 and cIAP2 in particular, prevent TNF-induced apoptosis and promote TNF-induced production of multiple cytokines and chemokines. Critically, we find that IAPs are required for TNF-induced cytokine production regardless of the cell death outcome. Therefore, IAPs are key regulators of TNF-induced inflammation in addition to their known role as regulators of TNF-induced cell death

IAPs also increase the threshold for TNF-induced necroptosis, when caspase activity is blocked. RIPK1 is widely believed to positively regulate necroptosis by phosphorylating RIPK3, leading to MLKL activation. Importantly however, we have

shown that RIPK1 functions to suppress necroptosis in response to TNF and toll like receptors. Indeed, we find that the RIPK1 kinase inhibitor Necrostatin-1 blocks necroptosis under conditions where RIPK1 knockdown potentiates the process. This indicates that the current interpretation of how Necrostatin-1 and RIPK1 participates in necroptosis requires re-evaluation. Our data suggest that kinase inactive RIPK1 (Necrostatin-1 inhibited), alters the function of RIPK1 to create an inhibitor of RIPK3 activation.

Necrosis is thought to promote inflammation through release of intracellular molecules called DAMPs, which are thought to have intrinsic pro-inflammatory properties. As necroptosis also results in the release of cellular constituents, this mode of cell death is thought to be detrimental *in vivo*, due to excessive DAMP-driven inflammation. However, we have shown that necroptosis attenuates the inflammatory potential of the triggering ligand, be it TNF or LPS, by terminating the production of multiple cytokines and chemokines.

Taken together, the unifying theme in this thesis is that interfering with molecules that regulate TNF-induced cell death, such as IAPs or caspase-8, also has dramatic consequences for inflammation and innate immunity. This aspect of TNF biology has remained relatively unexplored to date and is important to understand when investigating TNF- or microbe-driven pathology *in vivo*.

6.2 TNF-INDUCED CELLULAR OUTCOMES ARE TIGHTLY REGULATED

As discussed, TNF stimulation can result in either pro-inflammatory cytokine production or cell death, be it apoptosis or necroptosis. All of these outcomes have different impacts on the immune system. As a result, there is tight molecular regulation over these contrasting events. cIAPs appear to be critical in this context. Generally, cIAP1 and cIAP2 promote inflammation by ubiquitiinating RIPK1. This important event results in NFκB activation which promotes cell survival. If NFκB activation fails to initiate due to IAP loss, RIPK1 becomes a death-promoting

molecule. Indeed, RIPK1 and RIPK3 activity is tightly regulated by caspase-8. If caspase-8 activity is blocked, RIPK1 and RIPK3 associate in the necrosome to drive MLKL activation and necroptosis. Thus, cIAPs, RIPK1 and caspase-8 are critical in fine-tuning TNF-induced outcomes. Three key genetic observations strongly support this model. Firstly, cIAP1/2 double knockout is lethal, but can be rescued by loss of TNFR, suggesting that IAPs suppress TNF-induced cell death (Moulin et al., 2012). Secondly, RIPK1 knockout is lethal due to excessive TNF-induced apoptosis and necroptosis as a result of a failure to activate NFκB (Kelliher et al., 1998; Rickard et al., 2014). Thirdly, caspase-8 knockout is lethal due to excessive RIPK3-dependant necroptosis (Dillon et al., 2012). Therefore, the cIAP/RIPK1/Caspas-8 axis is of extreme importance both during development and for maintaining TNF signaling homeostasis during immune responses later in life.

6.3 IAP ANTAGONISTS SELECTIVELY SENSITIZE TUMOR CELLS TO APOPTOSIS BUT AFFECT INFLAMMATORY RESPONSES IN ALL CELL TYPES

IAP antagonists promote apoptosis by increased RIPK1 recruitment to FADD, leading to caspase-8 activation. However, cIAPs are also present in non-transformed cells suggesting that their removal would be equally toxic to healthy tissue. However, this does not appear to be the case, despite rapid cIAP degradation, increased RIPK1 recruitment and non-canonical NFκB activation (Vince et al., 2007). Indeed, cIAP1 knockout mice are normal with respect to TNFα-mediated NF-κB activation and cell death, demonstrating high selectivity for tumor cells. Certainly, it has long been realized that many but not all tumor cells are shifted in their response to death receptor engagement from a pro-inflammatory to a pro-cell death response. Indeed, TRAIL and Fas preferentially kill transformed cells but kill few primary cell types. The mechanism underlying this shift is not understood but it is likely to be overlapping with the propensity for IAP antagonists to also selectively kill tumor cells. Importantly, however, while IAP antagonism does not affect primary cell sensitivity to apoptosis, it does affect inflammatory pathways in these cell types, as discussed below.

6.4 IAPS AS THERAPEEUTIC TARGETS

We have shown that primary HUVEC cells are impaired in TNF-induced cytokine production but refractory to apoptosis upon IAP antagonism (Chapter 3). This characteristic of primary cells may create a therapeutic opportunity to exploit IAP antagonists as therapeutics for inflammatory diseases. Multiple chronic auto-inflammatory diseases including arthritis, inflammatory bowel disease and psoriasis are worsened by excessive TNF, as previously mentioned. Thus local IAP antagonism in these settings may ameliorate symptoms. A complicating factor may be the spontaneous release of chemokines such as RANTES, as we have demonstrated *in vitro* and *in vivo*. Attenuating multiple TNF-induced cytokines while increasing RANTES levels however, may still represent a beneficial outcome. As mentioned, some cancer cells require exogenous TNF in combination with an IAP antagonist to undergo apoptosis. In this regard, inflammatory stimuli have been used in combination with IAP antagonists with promising results. Indeed, it has been demonstrated that toll ligands such as Poly IC and CpG can synergize with IAP antagonist to cause tumor regression *in vivo* (Beug et al., 2014). This effect was due to toll ligand-induced TNF and IFN-β production, which increased tumor cell apoptosis in the presence of the IAP antagonist. In this setting, IAP loss from surrounding primary tissue may be a beneficial side effect, as it would prevent excessive cytokine production and immune cell infiltration.

6.5 DEVELOPMENT OF SPECIFIC IAP ANTAGONISTS

Because of the highly conserved sequence of mammalian IAP BIR domains, the current repertoire of IAP antagonists are unselective in which IAPs they bind to and degrade. Indeed the IAP antagonist used in this thesis, BV6, efficiently degrades cIAP1, cIAP2 and XIAP. Therefore, their use is limited as non-redundant IAP contributions in a particular process are hard to predict. Indeed, we have found that cIAP2 is the most important IAP in driving TNF-induced cytokine and chemokine production. However, a knockdown approach was required to elucidate this contribution. Interestingly, cIAP1 is known to regulate the protein levels of cIAP2 by conjugating K47-linked ubiquitin chains on cIAP2, leading to its proteasomal degradation (Conze et al., 2005). Consistent with the latter report, we found that

knockdown of cIAP1 lead to increased levels of cIAP2 (Figure 3.12). Therefore, it is not surprising that cIAP1 knockdown had little effect on TNF-induced cytokines, as it was compensated for by stabilization of cIAP2. As cIAP2 is not known to regulate cIAP1, knockdown of cIAP2 blunted TNF-induced cytokines. Therefore, it may be beneficial to develop IAP antagonists that specifically degrade cIAP2. Such an agent would likely blunt TNF-induced cytokine production as a therapeutic target, but may not activate non-canonical NFκB pathways causing spontaneous cytokine release. Indeed, an IAP antagonist specific to XIAP 'XIAP antagonist' has been shown to reverse B-cell lymphoma resistance to apoptosis (Cillessen et al., 2008). Similarly, because XIAP specifically ubiquitinates RIPK2 to drive NOD receptor-induced NFκB activation, XIAP antagonists may be therapeutically useful in dampening NOD receptor-induced inflammation during severe bacterial sepsis (Krieg et al., 2009).

6.6 EVOLUTION OF A REGULATED NECROSIS PATHWAY

Cell death is a crucial cellular response and is required for shaping tissues during development and removing unwanted or oncogenic cells later in life. Apoptosis was the first described regulated cell death process. It was found that the *C. Elegans* CED-9 protein regulated activation of the apoptosis executioners CED-3 and CED-4 (Hengartner et al., 1992). Indeed, CED3 and CED-4 were later revealed as homologues of human caspases and APAF-1 respectively (Yuan et al., 1993; Zou et al., 1997). Therefore, apoptosis represents an ancient and evolutionary conserved cell death mechanism, even in simple organisms. Interestingly, while CED-3 is a cell death inducing protease in *C. Elegans*, human caspase-1, -4 and -5 serve to promote inflammation, suggesting that cell death and immunity co-evolved as related processes. Apoptosis is now known to be a strategy to prevent viral replication. As a result, viruses have evolved to encode a plethora of inhibitors of caspase-8 activity, such as CrmA, to counteract death receptor-mediated apoptosis (Komiyama et al., 1994). Therefore, to counteract this viral strategy, it is believed that mammalian cells evolved the necroptosis pathway to eliminate cells infected with such viruses (Mocarski et al., 2012). Thus, cells treated with caspase inhibitors, such as zVAD, are thought to result in necroptosis by mimicking viral infection. If

necroptosis represents a host response to viral infection, designed to terminate viral replication by depriving the virus of a host, then it would be expected that viruses, being highly adaptive organisms would rapidly emerge that have lost their caspase inhibitory proteins. However, the fact that several viruses do encode caspase inhibitors strongly suggests that evoking necroptosis may be beneficial to certain viruses. This may be due to terminating inflammatory responses and aiding viral dissemination, as our data in chapter four suggests. Furthermore, there appears to be few inhibitors of necroptosis encoded by pathogen genomes. This suggests that either necroptosis is a relatively recent event in mammalian evolution, or alternatively, that it is not beneficial for pathogens to block necroptosis. What remains unclear, however, is whether RIPK3 and MLKL specifically evolved to drive necroptosis, or if they gained this function from a previous role in immune responses. This issue is discussed below.

6.7 RIPK3 KNOCKOUTS: DEFICIENT IN NECROPTOSIS, INFLAMMATION OR BOTH?

Many studies have utilized mice lacking RIPK3 as a model for necroptosis deficiency (Cho et al., 2009; Duprez et al., 2011; Upton et al., 2012). It has been reported that the detrimental effects of ethanol-induced liver damage, ischemia reperfusion, Cerulein-induced pancreatitis, as well as the lethal effects of systemic TNF are diminished in RIPK3 null animals (He et al., 2009; Linkermann et al., 2013; Roychowdhury et al., 2013). These observations have been widely interpreted to imply that necroptosis is a contributory factor in the tissue damage observed in all of these situations. However, these interpretations fail to take into account several key points. First, necroptosis is not typically observed in RIPK3 expressing cells unless caspase activation is inhibited and in the majority of studies using RIPK3 null animals, caspase inhibitors were not used. Therefore, the protective effects seen in RIPK3-deficient animals in various settings may be related to a direct role for RIPK3 in the regulation of inflammation, rather than its role in promoting necroptosis under conditions where caspase activation is blocked. In support of this concept, it has been demonstrated that RIPK3 deficient mice are protected from CLP-induced sepsis due to diminished infiltration of immune cells (Sharma et al., 2014). Indeed,

lower levels of cytokines and chemokines were detected in RIPK3 null animals compared to wild type animals. As no caspase inhibitor is present in this model, there is no reason to presume necroptosis would have occurred. It appears that RIPK3 is simply required for proper immune cell trafficking to inflammatory sites, independent of necroptosis (Sharma et al., 2014). Furthermore, if one assumes that RIPK3-dependent necroptosis does indeed occur *in vivo* under some circumstances, even in the absence of caspase inhibition, *RIPK3*$^{-/-}$ animals may manifest a more rapid recovery due to enhanced, as opposed to diminished, inflammatory responses. This interpretation is based on the observation that necroptosis is typically driven by death receptor (TNF, Fas) or toll-like receptor agonists (LPS, polyIC), all of which are potent instigators of pro-inflammatory cytokine production and inflammation. As we outlined earlier, if RIPK3-dependent necroptosis occurs in a cell that is responding to TNF or LPS by secreting a diverse array of pro-inflammatory cytokines, the pro-inflammatory program would be abruptly terminated. However, because *RIPK3*$^{-/-}$ null animals would be unable to under necroptosis in the same setting, the inflammatory response in these animals would be prolonged. Therefore *RIPK3*$^{-/-}$ animals may manifest a more robust inflammatory responses to pathogens or tissue damage which may promote more rapid resolution of the associated tissue disturbance to restore homeostasis.

As mentioned, RIPK3 can drive cytokine production in the absence of cIAPs (Wong et al., 2014). Furthermore, Tschopp and colleagues have demonstrated that IAPs function to repress RIPK3 dependant IL-1β maturation and release in macrophages following TLR priming (Vince et al., 2012). Removal of IAPs with a smac mimetic or via genetic intervention led to IL-1β secretion that was mediated by caspase-8, and not dependent on caspase-1 activity associated with the NLRP3 inflammasome. Reactive oxygen species (ROS) generated by RIPK3 kinase activity was shown to be necessary for this phenomenon, consistent with ROS being implicated as a driver of programmed necrotic death downstream of RIPK1 and RIPK3. Indeed, *in vivo* scenarios in which IAPs are lost simultaneously with TLR engagement are difficult to speculate upon, however IL-1β processing and release may be a useful reaction to such an event. This finding demonstrates that RIPK3 can function to drive inflammation, independent of DAMP release associated with necrosis.

It is likely, however, that other situations result in RIPK3-dependant cytokine production. Recently, RIPK3 has been identified as a regulator of LPS-induced cytokine production from dendritic cells. It was found that RIPK3 promotes tissue repair by regulating IL-22 production (Moriwaki et al., 2014). Therefore, using RIPK3 knockout animals as a strategy to assess the contribution of necroptosis to inflammation is problematic, as discussed above. MLKL however, has no identified role in signal transduction pathways leading to inflammation (Wu et al., 2013). Therefore, based on current knowledge, it may be more appropriate to use MLKL deficiency as a model of necroptosis deficiency. Ideally, experiments performed to investigate necroptosis-mediated inflammation should compare RIPK3 and MLKL deficiency, to isolate possible direct effects of either protein on inflammatory processes.

6.8 IMMUNOGENICITY OF NECROSIS VERSUS NECROPTOSIS

An important question to answer is whether the immune system can differentiate between a cell that has undergone RIPK3-dependent necrosis or weather the death was a result of a more rapid insult, perhaps physical damage. During apoptosis, phosphatidylserine is externalized on the plasma membrane, facilitating phagocytic recognition of apoptotic cells and promoting an anti-inflammatory response (Fadok et al., 1992). Furthermore, IL-33 among other molecules has been shown to be processed by caspase-3 and-7 during apoptosis to reduce its inflammatory potential (Luthi et al., 2009). Therefore, it is possible that downstream substrates of an activated RIPK3 pathway results in protein modifications representative of necroptosis. Such an event would inform the immune system that the necrotic death was associated with some level of control. RIPK3-dependent DAMP modification may allow a more intelligent reaction to the necrotic situation, leaving a signature of viral infection as opposed to physical injury. For example, injection of alum into the peritoneal cavity of mice has been shown to stimulate immune cell infiltration as a result of local necrosis and DNA release, creating an associated inflammatory cytokine profile (Marichal et al., 2011). RIPK3-driven necrotic debris may alter the

cytokine profile, promoting production of anti-viral molecules such as RANTES and Interferon.

It should also be considered that TNF rapidly induces an accumulation of intracellular chemokines prior to their export. This chemokine reservoir would be spilled into the extracellular milieu when the TNF signal finally translates to necrotic cell rupture. As sudden physical cell rupture in the absence of TNF stimulation does not allow time for chemokine accumulation, classic immune cell chemo-attractants such as IL-8, would not be present, outlining a simple difference in programmed necrotic immunogenicity. Certainly, it remains unexplored whether necroptotic cells have the same inflammatory potential as necrotic cells, and if this is altered during the programmed cell death process. Further studies will be required to resolve this issue.

6.9 PAMPS MAY CONTRIBUTE TO NECROPTOSIS-DRIVEN INFLAMMATION

Polly Matzinger introduced the 'danger hypothesis' in 1994, a model which is still relevant ot our understanding of innate immunity 20 years later. It was suggested that the immune system does not strive to recognize self from non-self, but merely to recognize signs of 'danger'. Danger would be represented by detection of Damage Associated Molecular Patterns (DAMPs), a sign of necrotic cell death. Indeed, Matzinger also proposed that detection of microbial products called Pathogen Associated Molecular Patterns (PAMPs), would not trigger a robust immune response in the absence of DAMPs. Thus, encountering PAMPs in the presence of DAMPs would be indicative of 'danger' and a robust inflammatory response would be initiated. While it now appears that PAMPs or DAMPs can promote inflammatory cytokine production independently of each other, it is likely that exposure to DAMPs and PAMPs results in a synergistic inflammatory response.

During necroptosis, membranes rupture and DAMPs are released. Depending on context, this may occur in the presence of PAMPs or it may occur in a sterile environment. Therefore, the magnitude of inflammation resulting from necroptosis *in vivo*, may depend on whether the trigger is a pathogen or an alternative endogenous, sterile stress. Indeed, necroptosis has been implicated in both viral and bacterial infection, but also in sterile models such as ischemic reperfusion injury and neuronal demise (Linkermann et al., 2013; Re et al., 2014). Knockout of caspase-8 or RIPK1 triggers necroptosis, which can be prevented by knockout of RIPK3. Therefore, RIPK3 null mice have been used to study the inflammatory effects of necroptosis *in vivo*. Indeed, the observed effects were attributed to DAMP release, such as IL-33 (Rickard et al., 2014). However, as barrier tissue breaks down in these mice, inflammation may be mostly attributable to pathogen invasion through damaged endothelium. Alternatively, it may be the result of a synergistic effect of DAMP exposure due to necroptosis, in the presence of PAMP detection, as Matzinger suggested (Matzinger, 1994).

6.10 Is the role of RIPK1 in necroptosis divergent between mouse and man?

The data in this thesis clearly demonstrates that knockdown of RIPK1 potentiates necroptosis in response to TNF, LPS and polyIC. This surprising result was found in murine L929 cells, murine RAW macrophages and murine embryonic fibroblasts. RIPK3 was identified in an siRNA screen for regulators of necroptosis induced by a combination treatment of TNF, IAP antagonist and zVAD in human HT-29 cells. Indeed, RIPK1 was required for RIPK3-dependent necroptosis in this setting and we have confirmed this result. Similarly, RIPK1 was required for necroptosis of human HaCaT keratinocytes, treated with Fas, smac mimetic and zVAD (Geserick et al., 2009). A major difference between these studies and our own, however, is that we have not used an IAP antagonist to sensitise L929, RAW or MEF cells to necroptosis, as it was not required (Chapter five). RIPK1 has been shown to be required for formation of the death inducing complex 'the Ripoptosome' following IAP depletion (Tenev et al., 2011). Therefore, it is possible that necroptosis of these cells requires a preformed Ripoptosome, provided by an IAP antagonist, and formation of this platform requires RIPK1. Indeed, few human cell lines can undergo

necroptosis in response to TNF and zVAD in the absence of an IAP antagonist, thus, this may explain the apparent discrepancy in the literature.

6.11 CONCLUSIONS

The data in this thesis support a number of conclusion, specifically that;

1. IAPs function to promote optimal TNF-induced cytokine production.
2. IAPs prevent spontaneous release of chemokines.
3. Necroptosis can suppress inflammation by attenuating TNF- or LPS-induced cytokine production, which is not compensated for by release of endogenous DAMPs.
4. RIPK1 is dispensable for necroptosis and can function as an inhibitor of the process.

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INFLAMMATORY CONSEQUENCES OF DIVERGENT CELL DEATH SIGNALS

Conor J. Kearney

Abstract

Throughout this thesis, TNF receptor stimulation and other innate immune receptors have been analyzed in the context of inflammation and cell death outcomes. While these two outcomes may initially appear separate and distinct processes, we have demonstrated that, in fact, it is important to study cell death and inflammation simultaneously. Three important questions were addressed; do IAPs influence TNF-driven inflammation independently of their role in cell death regulation? Does necroptosis diminish the inflammatory potential of the triggering ligand? Does RIPK1 function as a positive or negative regulator of necroptosis?

Members of the TNF superfamily, including TNF, can potently induce the production of pro-inflammatory cytokines, apoptosis or necroptosis, depending on cellular context. The IAPs and RIPK1 are critical in dictating whether TNF stimulation results in inflammation, apoptosis or necroptosis. Generally, it is believed that TNF receptor activation triggers recruitment of TRADD, RIPK1 and the IAPs. cIAP1 and cIAP2 then ubiquitinate RIPK1 to promote a signaling platform which leads to activation of multiple kinases. If RIPK1 fails to be ubiquitinated due to a block on NFkB activation or removal of IAPs, caspase-8 is activated, resulting in apoptosis. Importantly, we have shown that cIAP1 and cIAP2 in particular, prevent TNF-induced apoptosis and promote TNF-induced production of multiple cytokines and chemokines. RIPK1 is widely believed to positively regulate necroptosis by phosphorylating RIPK3, leading to MLKL activation. Importantly however, we have shown that RIPK1 functions to suppress necroptosis in response to TNF and toll like receptors. Necroptosis is thought to promote inflammation by release of DAMPS. However, we have shown that necroptosis attenuates the inflammatory potential of the triggering ligand, be it TNF or LPS, by terminating the production of multiple cytokines and chemokines. Taken together, the unifying theme in this thesis is that interfering with molecules that regulate TNF-induced cell death, such as IAPs or caspase-8, also has dramatic consequences for inflammation and innate immunity. This aspect of TNF biology has remained relatively unexplored to date and is important to understand when investigating TNF- or microbe-driven pathology *in vivo*.