Cell death signaling complexes during macrophage differentiation

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PREFACE

CONTRIBUTION OF COLLABORATORS

Dr. Robert Korneluk laboratory (University of Ottawa) provided the XIAP knockout mice and its corresponding wild type mice for the experiments.

APPROVALS

The experimental protocols used were approved by the University of Ottawa Animal Care Committee and include the protocols BMI1638 and BMI1639. A Biohazardous Materials Use Certificate was obtained from the University of Ottawa Office of Risk Management, Environmental Health and Safety.

ABSTRACT

Monocytes migrate to various tissues and differentiate to macrophages and mediate early control of pathogens. Various alternative pathways of cell death have been discovered which have been shown to play a key role in host survival. Herein, we investigated the impact of differentiation of monocytes to macrophages on their susceptibility to two distinct cell death inducing complexes, ripoptosome and necrosome. Our results indicate that differentiation of macrophages results in resistance to ripoptosome- but not necrosome-induced cell death. Additional experiments indicated that the resistance to ripoptosome signaling correlated with reduced caspase activation and enhanced expression of anti-apoptotic mediators XIAP and $cFLIP_{L.}$ Our results also reveal the contradictory roles of p38 MAPK/MK2 in stimulating (phosphorylating RipK1) or inhibiting (reducing TNF-α expression and caspase 8 activation) ripoptosome signaling. These findings reveal novel mechanistic insights that can be exploited for development of therapeutics.

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LIST OF ABBREVIATIONS

AIF	Apoptosis-Inducing Factor
APAF-1	Apoptotic Protease Activating Factor
AP-1	Activator Protein 1
ATF-1/ATF-2	Activating Transcription Factor 1/ Activating Transcription Factor 2
ATP	Adenosine Triphosphate
BAD	Bcl-2-associated death promoter
BAFF	B-cell-Activating Factor
BAFF-R	B-cell-Activating Factor of the TNF family Receptor
BAK	Bcl-2 homologous antagonist/killer
BAX	BCL-2 Associated X protein
BCL-2	B-Cell Lymphoma Protein-2
BCL-XL	B-Cell Lymphoma-extra large
BCL-XS	B-Cell Lymphoma-extra small
BID	BH3 (Bcl-2 homology 3) interacting-domain death agonist
BIR	Baculoviral IAP Repeat
BMDM	Bone Marrow Derived Macrophage
BP	Birinapant
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CARD	Caspase Activation and Recruitment Domain
cFLIP	Cellular FLICE(caspase-8)-like inhibitory protein
cIAP (1/2)	Cellular Inhibitor of Apoptosis (1/2)
CLL	Chronic Lymphocytic Leukemia
CMoPs	Common Monocyte Progenitors
CREB	cAMP Response Element-Binding Protein
CSF-1	Colony Stimulating Factor-1
CYLD	Cylindromatosis
DAMP	Damage Associated Molecular Pattern
DAI	DNA-dependent Activator of IFN-regulatory factors
DD	Death Domain
DC	Dendritic Cell
DED	Death Effector Domain
DIABLO	Direct IAP-Binding Proteinwith Low pI
DISC	Death-inducing signaling complex
ERK	Extracellular signal–regulated kinase
FADD	FAS-associated protein with death domain
FBS	Fetal Bovine Serum
FLIP	FLICE-Like Inhibitory Protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HSP 27	Heat Shock Protein 27
IAP	Inhibitor of Apoptosis Protein
IBD	Inflammatory Bowel Disease
IBM	IAP Binding Motif
IFN	Interferon
IFN-α	Interferon-Alpha

IFN-β	Interferon-Beta
IFNAR1	Interferon α/β Receptor 1
IFN-I	Type I Interferon
IkB	Inhibitor of kappa B
IKK	Inhibitor of Nuclear Factor Kappa-B Kinase
IL-1/IL-6	Interleukin-1/Interleukin-6
IL-1R	Interleukin-1 Receptor
IP	Immunoprecipitation
IRF	Interferon Regulatory Factor
ISG	Interferon Stimulated Gene
ISGF3	Interferon Stimulated Gene Factor 3
JNKs	c-Jun N-terminal kinases
KO	Knockout
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeats
LSP1	Lymphocyte-Specific Protein 1
LT-βR	Receptor for lymphotoxin-β
LTβ	Lymphotoxin-Beta
LUBAC	Linear ubiquitin chain assembly complex
MAPKAPKs	MAP Kinase Activated Protein Kinase
MAPKs	Mitogen-activated protein kinases
MCMV	Murine Cytomegalovirus
M-CSF	Macrophage Colony Stimulating Factor
MDP	Macrophage and Dendritic cell Precursor
MEK	MAPK/ERK kinase
MEKK2	MEK kinase 2
MFI	Mean fluorescence intensity
MK2/3	MAP Kinase-activated protein Kinase 2 or 3
MLKL	Mixed Lineage Kinase domain-Like Protein
MPS	Mononuclear Phagocytic System
MPT	Mitochondrial Permeability Transition Pore
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MyD88	Myeloid Differentiation Primary Response Gene 88
NEMO	NF-κB Essential Modulator
NFκB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NIK	NF-kB-Inducing Kinase
NOD	Nucleotide-binding Oligomerization Domain
p38 MAPK	p38 Mitogen-Activated Protein Kinase
PAMP	Pathogen Associated Molecular Pattern
PARP	Poly ADP Ribose Polymerase
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PI	Propidium Iodide
PolyI:C	Polyinosinic-polycytidylic Acid
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene fluoride

RANK	Receptor Activator of NF-kB
RANKL	Receptor Activator of NF-kB Ligand
RBC	Red Blood Cell
R8	RPMI with 8% Fetal Bovine Serum
RHIM	Rip Homotypic Interaction Motif Domain
RHD	Rel-Homology Domain
RIG-I	Retionic acid-Inducible Gene I
RIPK1	Receptor Interacting Protein Kinase 1
RIPK3	Receptor Interacting Protein Kinase 3
RING	Really Interesting New Gene
RLU	Relative Luminescence Unit
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPM	Revolutions per minute, a measure of the frequency of rotation
RPMI	Roswell Park Memorial Institute Medium
SAPK	Stress-Activated Protein Kinase
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematous
SM	SMAC mimetic
SMAC	Second Mitochondria-derieved Activator of Caspases
ST	*Salmonella enterica* serovar Typhimurium
TAB2	Transforming Growth Factor-Beta Activated Kinase 2 Binding Protein
TACE	Tumor Necrosis Factor-Alpha Converting Enzyme
TAK1	Transforming Growth Factor-Beta-Activated Kinase 1
tBID	Truncated BID
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween
TIR	Toll-interleukin 1 Receptor
TLR	Toll Like Receptor
TLR-3	Toll like receptor 3
TLR-4	Toll like receptor 4
TNF-α	Tumor Necrosis Factor-alpha
TNFR	Tumor Necrosis Factor Receptor
TNF-R1	Tumor Necrosis Factor Receptor 1
TNF-R2	Tumor Necrosis Factor Receptor 2
TRADD	TNFR Associated Death Domain Protein
TRAF1/2/3	TNFR Associated Factor 1 or 2 or 3
TRAIL	TNF Related Apoptosis Inducing Ligand
TRAIL-R1/2	TNF Related Apoptosis Inducing Ligand-Receptor 1/2
TRIF	TIR-domain Containing Adapter Inducing IFN-ß
T1D	Type-1 Diabetes
TWEAK	TNF-related Weak Inducer of Apoptotis
UBA	Ubiquitin Associated Domain
UV	Ultraviolet
vFLIP	Viral Inhibitory Protein FLICE-Like Inhibitory Protein

vICA	Viral Inhibitor of Caspase 8-induced Apoptosis
vIRA	Viral Inhibitor of Rip Activation
WT	Wild-Type
XIAP	X-linked inhibitor of apoptosis protein
zVAD	Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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1.0. INTRODUCTION

Cell death is a fundamental mechanism that plays a pivotal role in the life of all living organisms. It plays an important role in maintaining tissue homeostasis, organ development, and in balancing the life's physiological processes (Pasparakis and Vandenabeele, 2015). Cell death has been studied for a long time, however the programmed cell death and its role in inflammation and disease progression have been in the limelight since few decades (Pasparakis and Vandenabeele, 2015). Cell death of various immune cells such as macrophages and dendritic cells is one of the mechanisms through which intracellular pathogens are controlled at an early stage. Any deviation in the mechanisms that control cell death is associated with many pathological diseases. For example, enhanced cell death is associated with neurogenerative disorders and chronic inflammatory diseases, and insufficient cell death is associated with cancers, autoimmune diseases and chronic infections (Danial and Korsmeyer, 2004; Dong et al., 2015).

Apoptosis is considered as a physiological form of programmed cell death, which doesn't evoke any inflammatory response (Elmore, 2007). Various alternative forms of cell death have been discovered recently, which are highly inflammatory (Pasparakis and Vandenabeele, 2015). Engagement of inflammasomes has been shown to induce cell death by pyroptosis (Nagata and Tanaka, 2017), and activation of necrosomes has been shown to result in cell death by necroptosis (Pasparakis and Vandenabeele, 2015). Recently a novel cell death-inducing complex has been discovered which is termed as the "Ripoptosome". This cell death platform is assembled in response to gentotoxic stress or depletion of the endogenous inhibitors of apoptosis proteins (cIAPs) (Tenev et al., 2011).

Cell death mechanisms appear to play an important role in the immune system, and the impact of the various forms of cell death on different types of immune cells is not clear.

Monocytes are the front line cells of the immune system that circulate through the blood and differentiates into macrophages following stimulation. In this thesis I have interrogated the impact of macrophage differentiation on their susceptibility to cell death by the ripoptosome and necrosome signaling platforms.

1.1. The Immune system

Immune system is efficient at recognizing any foreign substances, which results in its elimination and consequent protection against danger. The discovery of immune system dates back to 18th century when Edward Jenner introduced the concept of vaccination against human smallpox (Janeway et al., 2005). Elie Metchnikoff discovered macrophages as a phagocytic cell and its role in innate immunity. In the meantime, the discovery of vaccines against cholera and rabies by Louis Pasteur and Koch's postulates on host pathogen interactions set new victories against deadly infections. All these discoveries led to a deeper understanding of the immune system in our body and paved a new path for the immunological research (Janeway et al., 2005).

Microorganisms and the metazoan hosts have been living in close association since ages. Due to the evolution under the selective pressure of microorganisms, the immune system in multicellular organisms are now equipped with vast array of cells, molecules and structures for the recognition of diverse pathogens and prevention of infections (Medzhitov, 2007).

The immune system can be broadly categorized into the innate and adaptive immune system. The innate immune system acts as a first line of defense against any infectious agents, which becomes alerted within minutes to hours of pathogen entry. Innate immune cells are equipped with germ line encoded receptors called pattern recognition receptors

(PRRs), such as toll-like receptors (TLRs), NOD-like receptors and RIG-1-like receptors that are efficient in recognizing pathogen associated molecular patterns (PAMPs) of the microbes (Medzhitov, 2007). PAMPs are the invariant, conserved structure or molecules shared by a wide variety of microorganisms. Lipopolysachharide (LPS), peptidoglycan and lipotechchoic acids are the common cell surface PAMPs in bacteria (Medzhitov, 2007). On recognition of PAMPs by PRRs, the innate immune cells such as macrophages, dendritic cells, monocytes and neutrophils become activated. The anti-microbial responses become enhanced through different mechanisms such as phagocytosis, opsonization, production of pro-inflammatory cytokines, expression of anti-microbial mediators, initiation of inflammation and induction of cell death (Medzhitov, 2001; Medzhitov, 2007).

Adaptive immunity comes into play after few days of infection. Adaptive immunity is initiated by antigen-presentation by macrophages or dendritic cells to T cells. T cells have a large repertoire of antigen receptors formed from the somatic recombination of their respective encoding genes and are highly specific for each antigen (Parkin and Cohen, 2001). On microbial antigen encounter, T cells ($CD4^+$ T cells and $CD8^+$ T cells) and B cells undergo clonal selection and expansion and differentiate into antigen specific T effector cells and antibody secreting cells respectively (Parkin and Cohen, 2001). This forms an army of antigen specific cells that mount a selective immune response against that pathogen. One of the hallmarks of the adaptive immunity is the generation of immunological memory against the previously encountered pathogen. Thus on re-exposure to the same pathogen, there is a rapid and robust adaptive immune response against that specific pathogen, which provides effective pathogen clearance and long-term immunity to the host (Janeway et al., 2005).

1.2. Monocytes and macrophages

The pluripotent hematopoietic stem cell in the bone marrow is the origin of the majority of the leukocytes in the body. The mononuclear phagocytic system (MPS) is the cell lineage that originates from the hematopoietic stem cell in the bone marrow and is the precursor of monocytes, macrophages and dendritic cells (Gordon and Taylor, 2005). MPS give rise to mature monocytes with various sequential stages of differentiation including monoblast and pro-monocyte (Gordon and Taylor, 2005). Monocytes circulate in the blood for a few days and differentiate into macrophages or dendritic cells (DCs) in the tissues in response to chemokines or cytokines (Ginhoux and Jung, 2014). Some recent evidences have identified macrophage and dendritic cell precursor (MDP) as the bone marrow precursors of DCs and monocytes (Varol et al., 2007) and common monocyte progenitors (CMoPs) as the specific bone marrow precursor of monocytes (Hettinger et al., 2013).

Monocytes represents approximately 10% of the leucocytes in human blood (Das et al., 2015). These circulating blood leucocytes have multiple roles in immune response. They maintain the homeostasis of tissues by replenishing the pool of tissue macrophages in the steady state. They are efficient in mounting an effective immune response during infection against wide range of micro-organisms or any injury or inflammation (Shi and Pamer, 2011). In the meantime these monocytes become the source of macrophages at the site of inflammation (Ginhoux and Jung, 2014). They are important in various diseases such as cardiovascular diseases, Type 1 diabetes and cancer (Hettinger et al., 2013).

Macrophages are the large mononuclear innate immune cells, which are present in all the tissues of the body. Russian-French biologist, Ellie Metchnikoff discovered the phagocytic cell in 1884 and named it as macrophages (Janeway et al., 2005). They are long-lived cells with a limited proliferative capacity and act as a first line of defense against

invading pathogens. Macrophages sense the pathogens with the help of their PRRs and enhance their anti-microbial responses through different mechanisms such as phagocytosis, production of pro-inflammatory cytokines, expression of anti-microbial mediators and induction of cell death (Janeway et al., 2005). Besides being the effector cells for mediating inflammation, macrophages also play an important role in resolution of inflammation by remodeling and repairing the tissues through efferocytocis (phagocytosis of the apoptotic bodies) (Gordon and Taylor, 2005).

Macrophages are highly heterogeneous group of cells whose physiology and function are dependent on its anatomical location. For example they are termed as osteoclasts in the bone, alveolar macrophages in the lungs, kupffer cells in the liver, microglial cells in the brain and peritoneal macrophages in the peritoneal cavity (Gordon and Taylor, 2005). It has been known that most of the tissue macrophages are derived from the blood circulating monocytes. There are recent reports which show that the embryonic precursors are the source of most of the tissue macrophages, which undergo self-replication and are not dependent on replenishment of the macrophage pool by the circulating monocytes (Schulz et al., 2012; Yona et al., 2013). However, in the context of inflammation, most of the studies have shown that monocytes are the primary source of the macrophages (Gordon and Taylor, 2005).

The differentiation of monocytes to macrophages is believed to be highly ambiguous. Different cell lines at various stages of differentiation of monocytes are often used to study the physiology and function of a tissue macrophage. It is hard to say whether the macrophages that are formed in the tissue after the differentiation of monocytes are the terminally differentiated cells (Gordon and Taylor, 2005)..

1.3. Inflammation

Inflammation is a host response to any tissue injury, microbial infection, cellular stress or stimuli (Medzhitov, 2008). Macrophages are the front line cells of the immune system that are responsible for the initiation, maintenance and resolution of inflammation. During infection, injury or tissue damage, macrophages sense the PAMPs or endogenous damage associated molecular patterns (DAMPs) present in the damaged tissues and initiate the cascade of signaling pathways culminating into the production of different chemokines, pro-inflammatory cytokines, histamines, leukotrienes and prostaglandins. These mediators of inflammation causes the vasodilation of surrounding blood vessels, resulting into the increased blood flow to the site of infection and consequent infiltration of a large number of neutrophils and monocytes to the site of infection (Ashley et al., 2012; Medzhitov and Horng, 2009). The neutrophils at the site of infection secrete various toxic components such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) to eliminate engulfed pathogens. In this scenario, most of the host tissues are also subjected to a collateral damage (Medzhitov, 2008). These interactions result into the typical four cardinal symptoms of inflammation namely calor (heat), tumor (swelling), rubor (redness) and dolor (pain) (Fullerton and Gilroy, 2016; Medzhitov and Horng, 2009). The acute inflammation phase is followed by the repair phase wherein tissue resident macrophages mediate repair and remodeling of tissues resulting in the resolution of the inflammation (Fullerton and Gilroy, 2016). If the acute inflammation is unable to clear the pathogens and the pathogens persist, it results in a state of chronic inflammation (Medzhitov, 2008).

Inflammation can be considered as a double-edged sword. Although inflammatory response is a natural phenomenon that ensures physiological balance, it must be tightly regulated because uncontrolled inflammation can cause various pathological conditions such

as septic shock, rheumatoid arthritis, diabetes and multiple sclerosis (Ashley et al., 2012; Medzhitov, 2008). Recently, various forms of programmed cell death such as necroptosis and pyroptosis have been shown to promote inflammatory responses through the processing of IL-1 family of cytokines and cell rupture which results in the release of numerous inflammatory DAMPs (Pasparakis and Vandenabeele, 2015).

1.3.1. TLR signaling

Toll like receptors (TLRs) are the evolutionarily conserved receptors predominantly present in immune cells such as the macrophages, neutrophils and dendritic cells (Medzhitov, 2001) that can activate various signaling processes for the induction of pro-inflammatory cytokines, interferons (IFNs) and other mediators for effective immune response (Kawasaki and Kawai, 2014). TLR signaling plays an important role in innate immunity as well as adaptive immunity (Medzhitov, 2001). TLR4 is the first TLR discovered in mammals, which is the receptor for lipopolysaccharide (LPS), an outer membrane component of all gram-negative bacteria (Neill et al., 2013). TLRs are type-1 integral membrane glycoproteins which are grouped under the interleukin-1 receptor (IL-1Rs) superfamily (Akira and Takeda, 2004). They have Leucine-rich repeats (LRRs) in their extracellular domain which resembles the shape of a horse shoe and mediates recognition of PAMPs. On the cytoplasmic domain TLRs have Toll/IL-1R (TIR) domain that mediates downstream signaling (Akira and Takeda, 2004; Kawasaki and Kawai, 2014). TLRs are either present in the cell surface or in the intracellular compartments such as the endosomes, lysosomes or endoplasmic reticulum (Kawasaki and Kawai, 2014). Upon engagement of TLRs by their ligands, TLRs get dimerized, which results in their conformational change. The cytoplasmic domain then recruits the adapter proteins to initiate the signaling cascade.

MyD88 is an adaptor protein in TLR signaling and MyD88 pathway is important for the induction of pro-inflammatory cytokines and other anti-microbial mediators through the activation of MAPKs signaling and NF-kB activation (Kawasaki and Kawai, 2014).

Besides MyD88 pathway, TLR signaling also induces TRIF pathway, which is activated in response to TLR4 and TLR3 signaling but not induced by other TLR pathways (Kawasaki and Kawai, 2014). TRIF pathway is reported to induce IFN-β production and the expression of other IFN-inducible genes through the activation of interferon-regulatory factors (IRFs). IRFs are the family of transcription factors that induce type 1 IFNs (Akira and Takeda, 2004).

1.3.2. Cytokine signaling

Cytokines play a vital role in tissue homeostasis, immunity and inflammation. TNF-α is one of the cardinal pro-inflammatory pleotropic cytokines expressed by a vast array of cells but mostly expressed by the immune cells such as activated monocytes/macrophages, activated NK cells and T cells (Sedger and Mcdermott, 2014). TNF-α is involved in many physiological aspects and anti-microbial responses including the induction of the pro-inflammatory signaling, cell differentiation, cellular communication, host defense and cell death (Brenner et al., 2015). Primarily, TNF-α is expressed as a transmembrane protein, which is then proteolytically cleaved into its trimeric soluble form by an enzyme TACE (Brenner et al., 2015; Sedger and Mcdermott, 2014). The trimeric TNF binds to its trimeric receptors TNF-R1 or TNF-R2 to initiate the signal transduction. TNF-R1 is expressed in all types of cells while TNF-R2 are mostly restricted to the immune cells and the endothelial cells. Both forms of TNF-α can activate TNF-R1 while TNF-R2 has been reported to be activated primarily by transmembrane TNF-α (Kalliolias and Ivashkiv, 2016). Once TNF-α

binds to TNF-R1, there is a conformational change in the cytoplasmic death domain (DD) of TNF-R1, which enables it to recruit adaptor protein Tumor necrosis factor receptor type 1-associated DEATH domain (TRADD) to initiate different signaling complexes having distinct functional outcomes. TNF-α can induce inflammatory responses as well as programmed cell death following engagement of the receptor, suggesting that the outcome of a given cytokine-receptor is dependent on the propensity of the cells to congregate various cell survival or cell death pathways (Pasparakis and Vandenabeele, 2015). On the other hand, TNF-R2 are devoid of DD and thus signals through TRAF1 or TRAF2 for NF-kB activation. It is interesting that TNF-R2 doesn't signal for cell death induction, and is more inclined towards cell survival and tissue homeostasis (Brenner et al., 2015). TNF-α acts as a powerful cytokine against viruses and bacteria by inducing various mediators for recruitment of innate immune cells, induced NF-kB mediated differentiation and maturation of myeloid cells and direct cell death of virus infected cells (Sedger and Mcdermott, 2014).

TNF-α has been associated with many autoimmune and inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease (IBD) (Crohn disease and ulcerative colitis), septic shock, systemic lupus erythematous (SLE), psoriasis, multiple sclerosis and type 1 diabetes (T1D) (Brenner et al., 2015). Many approaches have been made to target TNF-α for the generation of effective therapeutics against these diseases. The cytostatic activity of TNF-α has been proposed to be exploited in some cancer therapies, however due to its high toxicity and potential to induce carcinogenesis and mutagenesis in tumors, its use has been limited (Aggarwal, 2003; Kalliolias and Ivashkiv, 2016).

Interferons (IFNs) are the group of widely expressed cytokines, which are important in anti-viral responses, immune-surveillance of malignant cells and induction of cell death

(Mcnab et al., 2015; Platanias, 2005). IFNs has been classified into three families, type I IFN, type II IFN and type III IFN. Type I IFN consist of many IFNs of which IFN-α and IFN-β are the well characterized and studied IFNs till date. The type I IFNs produced, bind to their cognate receptor IFN-α/β receptor (IFNAR) and initiate the signal transduction for the transcription of many IFN-stimulated genes (ISGs). They can signal in an autocrine or paracrine fashion. (Ivashkiv and Donlin, 2014). One of the main functions of IFN signaling is to confer anti-viral responses by interfering with the viral replication cycle and inducing an anti-viral state in the infected as well as uninfected neighboring cells (Mcnab et al., 2015). Recently, IFNAR1 signaling has been reported to be indispensable in necroptotic cell death (McComb et al., 2014).

In retrospect, cytokines such as TNF-α and IFN-β have multiple functions in immunity. They can act like a double-edged sword, which may confer protection against microbes but in the meantime may have deleterious consequences to the host.

1.3.3. Downstream signaling

The innate immune cells such as macrophages and dendritic cells recognize the microbial PAMPs or DAMPs and initiate downstream signaling cascades for the activation of kinases (MAPKs) and transcription factors such as NF-kB, AP-1, and interferon regulatory factor (IRF) transcription factors. These transcription factors induce the transcription of numerous genes that are responsible for pro-inflammatory response and pathogen clearance (Newton and Dixit, 2012). However, the timely resolution of these responses is important in order to avoid the detrimental effects to the host (Medzhitov and Horng, 2009). The deeper understanding of the transcriptional program is necessary to target

the critical components of the inflammatory response for the development of therapeutics against chronic inflammatory diseases.

1.3.3.1. Canonical NF-kB pathway

NF-kB activation results from two distinct pathways, the canonical pathway and the non-canonical pathway. Mammalian system consists of five NF-kB members RelA (p65), RelB, c-Rel, and the precursor proteins p105 (NF-κB1) and p100 (NF-κB2), that undergo processing to form p50 and p52 respectively. All of these members have an N-terminal Rel-homology domain (RHD) that are important in DNA binding, dimerization and nuclear translocation (Shih et al., 2010). Different complexes are formed from the homo- or hetero-dimerization of these different members of NF-kB among which RelA/p50, RelB/p52 and c-Rel/p50 are the most important ones for physiological processes (Shih et al., 2010). In the steady state, the NF-kB dimers are retained in the cytoplasm in an inactive state by the inhibitors of NF-kB (IkBs) (Perkins, 2007). The canonical pathway is activated in response to the PAMPs, DAMPs or cytokines. The signals from these stimulators converge in the activation of IKK complex, culminating in the activation of canonical pathway of NF-kB (Gerondakis et al., 2013). In case of TNF signaling, the binding of TNF-α to its receptor TNF-R1 induces the recruitment of TNFR-associated death domain protein (TRADD) which further recruits receptor interacting protein kinase 1 (RipK1), TNFR-associated factor 2 (TRAF2) and cellular inhibitors of apoptosis proteins 1/2 (cIAP1/2) to the receptor on its cytoplasmic domain and forms the TNF-R1 complex I (Brenner et al., 2015). cIAP1/2 are the E3 ubiquitin ligases that mediate K63 linked poly-ubiquitination of many components of the complex I. Most importantly, the K63-linked poly-ubiquitination of RipK1 by cIAPs

positively regulates the canonical NF-kB activation pathway. RipK1 is further stabilized by the M1-linked ubiquitination, which is mediated by the linear ubiquitin chain assembly complex (LUBAC). LUBAC is recruited to the complex after RipK1 is held in complex I by ubiquitination by cIAPs (Brenner et al., 2015). These ubiquitination linkages on RipK1 further results in the recruitment of TGF-beta activated kinase 1 binding protein 2 (TAB2) and transforming growth factor-beta-activated kinase 1 (TAK1) to the receptor complex which mediate activation of the IKK complex. The activated IKK complex, consisting of IKK proteins and NEMO, induces phosphorylation and proteasomal degradation of IkB, which is bound to the NF-kB heterodimer (p50/RelA) in the cytoplasm. The degradation of IkB releases the p50/RelA heterodimer from its inactive state, which then translocates to the nucleus. In the nucleus, the transcription factor induces transcription of many NF-kB target genes (Oeckinghaus et al., 2011).

1.3.3.2. Non-canonical NF-kB

Non-canonical NF-kB is activated by stimulation of TNF receptor superfamily members CD40, lymphotoxin-β receptor (LTβR), B-cell-activating factor of the TNF family receptor (BAFF-R) and receptor activator of NF-kB (RANK) by their respective ligands CD40L, LTβ, BAFF and RANKL (Perkins, 2007; Shih et al., 2010).

In the steady state, cIAPs, TRAF2 and TRAF3 regulates non-canonical NF-kB through constitutive K48-linked ubiquitination and proteasomal degradation of NIK, a member of MAP3 kinase family (Fulda and Vucic, 2012). The degradation of cIAPs by SMAC/SMAC mimetic or through TNF-related weak inducer of apoptosis (TWEAK) stimulation stabilizes NIK. The stabilization and accumulation of NIK protein activates IKKα and induces phosphorylation and proteasomal processing of p100 to p52 by 26S

proteasome, thus activating the p52/RelB dimers (Gerondakis et al., 2013; Shih et al., 2010). The dimers then translocate to the nucleus and transcribe various NF-kB target genes (Shih et al., 2010).

Many inhibitors of cIAPs were found to activate the non-canonical NF-kB suggesting cIAPs having a major role in regulating non-canonical NF-kB along with TRAF3 (Zarnegar et al., 2008). Thus cIAPs are considered as the key player in NF-kB activation that positively regulate canonical NF-kB while suppress the activation of non-canonical NF-kB (Tenev et al., 2011).

1.3.3.3. MAP kinases (MAPKs)

Multicellular organisms respond to external stimuli by various mechanisms among which activation of different kinases is one of the predominant mechanisms. Highly conserved mitogen-activated protein kinases (MAP kinases/MAPK) are involved in transmitting the extracellular stimuli to various targets within the cell and promote cell survival, cell proliferation, gene expression, cell differentiation and cell death (Strniskov et al., 2002). The MAPK cascade involve three modules, first is the activation of upstream MAPK/ERK kinase (MEK kinase) (MEKK or MAPKKK), which phosphorylates and activates the second kinase, MAPK kinase (MEK, MKK or MAPKK), which further phosphorylates the downstream kinase, the MAPK, on threonine and tyrosine residues. The MAPKs are the serine/threonine kinases that phosphorylate the substrates targeting different cellular processes (Chang and Karin, 2001; Strniskov et al., 2002). In the mammalian system, the three major MAPK cascades are known, extracellular signal-related protein kinases (ERK1/2) cascade, Jun amino-terminal protein kinases (JNK/SAPK) cascade and p38 MAPK (p38α/β/γ/δ) cascade (Chang and Karin, 2001).

ERK1/2 is the first MAPK pathway studied and is activated by various stimuli such as mitogens, hormones and many internal processes. ERK proteins are made up of two genes erk1 and erk2 (Shaul and Seger, 2007). Phosphorylation at Threonine and Tyrosine residues of the ERK proteins results in their activation, which culminates in ERK mediated phosphorylation of numerous substrates in the cell cytoplasm or in the cell nucleus. ERKs are known to activate a vast array of transcription factors such as c-Fos, cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), NF-kB/ IkBα, ELK1, p53 and Ets1/2, and control many cellular processes such as transcription, translation, cell differentiation and apoptosis (Shaul and Seger, 2007; Strniskov et al., 2002). ERK has been reported to be a positive as well as negative regulator of apoptosis depending on the cell type and cellular microenvironment (Strniskov et al., 2002).

JNK pathway, also known as SAPK pathway is known to be activated by various kinds of genotoxic as well as environmental stresses such as UV radiation, TNF, chemical stress, heat shock and is associated with inflammation, cell differentiation, cell survival, cell migration and apoptosis (Strniskov et al., 2002; Wagner and Nebreda, 2009). JNK proteins have many isoforms among which JNK1 and JNK2 are widely expressed in cells. JNK induces phosphorylation-mediated activation of many transcription factors such as c-Jun and AP-1 and other substrates such as ATF-2, ELK1 and BCL-2 (Strniskov et al., 2002). JNKs can function in pro-apoptotic signaling by the mitochondrial pathway as well as in the anti-apoptotic pathway by the transcriptional activation of AP-1 leading to cell proliferation. These contradictory apoptotic functions are dependent mostly on the strength of the signal. (Wagner and Nebreda, 2009).

p38 MAPK was first identified as a 38 kDa protein that was phosphorylated on tyrosine in response to LPS stimulation in macrophages (Han et al., 1993). There are four

different members of p38 MAPKs: p38α, p38β, p38γ and p38δ among which p38α and p38β are ubiquitously expressed in tissues while p38γ and p38δ are tissue specific (Zarubin and Han, 2005). p38α is highly expressed in cells and has been reported to be activated in different cells in response to inflammation, indicating its significant role in inflammatory responses. p38 MAPK is known to be activated by a variety of stimuli including UV light, heat, osmotic shock, pro-inflammatory cytokines (TNF-α and IL-1), protein synthesis inhibitors, H_2O_2 and growth factors (CSF-1) (Strniskov et al., 2002; Zarubin and Han, 2005). The upstream activator kinases of p38 MAPK are the MEK 3, 4 and 6. The downstream substrate for p38 MAPK is MAP kinase-activated protein kinase 2 and 3 (MK2 and MK3). MK2 or MK3 are known to activate many downstream proteins such as heat shock protein (HSP 27), lymphocyte-specific protein 1 (LSP1), CREB transcription factor and activating transcription factor 1 (ATF1) (Zarubin and Han, 2005). MK2 was reported to be an important downstream kinase of p38α activation for the production of TNF-α in response to LPS stimulation (Kotlyarov et al., 1999). Activation of p38α has been implicated in tumor suppression through induction of apoptosis, negative regulation in cell proliferation and terminal cell differentiation. On the contrary, many reports have shown its role in cancer progression, angiogenesis and cell proliferation through the production of different cytokines (TNF, IL-1 and IL-6), which has pro-survival and angiogenic effects (Wagner and Nebreda, 2009). Since, many types of cancers rely on p38α activity, novel p38α inhibitors have been developed recently in order to treat tumors by sensitizing the cells to apoptotic cell death. This dual role of p38 in pro-apoptotic and anti-apoptotic pathways is dependent on cell type, strength and duration of the signal, the cellular microenvironment and the crosstalk with other pathways (Wagner and Nebreda, 2009).

1.4. Cell death and inflammation

The concept of cell death emerged in 1842 when Karl Vogt demonstrated that cell death is important for removing the notochord for the formation of vertebrae of the midwife toad during metamorphosis (Clarke and Clarke, 1996). Since then, cell death was shown to be an important and indispensable mechanism that was responsible for the survival of living organisms. Later on during 1960s and 1970s, the concept of programmed cell death (PCD) emerged with the development of electron microscopy and the new histological techniques. During the years of 1989 to 1991, various programmed cell death genes such as Bcl-2, FAS and p53 were identified that provided genetic basis of PCD (Lockshin and Zakeri, 2001). The programmed cell death was found to be important for the normal development, maintenance of homeostasis of the body and in promoting the removal of self-reactive immune cells (Fink and Cookson, 2005). This form of cell death was termed apoptosis with distinct features of chromatin condensation, shrinkage of cells and nuclear fragmentation. On the other hand, the accidental cell death in response to excessive trauma or injury was termed necrosis (Lockshin and Zakeri, 2001). This form of cell death was often found to be associated with highly virulent infections.

Till date, many forms of programmed cell death have been elucidated such as apoptosis, necroptosis, pyropotosis, ferroptosis, parthanatos, pyronecrosis and NETosis. Cell death induced during apoptosis and pyroptosis has been shown to be mediated by caspases, the cysteine-aspartic proteases, whereas cell death during necroptosis is mediated by caspase independent mechanisms (Pasparakis and Vandenabeele, 2015). Disruption of PCD can have deleterious effects on the host. The role of PCD in inflammatory responses has been an area of active investigation for the last few decades. Apoptosis has been considered as a developmental and non-inflammatory mechanism of cell death while pyroptosis and

necroptosis have been considered as highly immunogenic forms of cell death due to the membrane lysis of dying cells which results in the release of numerous DAMPs (Yang et al., 2015). Recent reports have highlighted the role of necroptosis in the pathogenesis associated with various chronic inflammatory diseases in humans such as inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis and alcohol-induced liver disease. The release of DAMPs following necroptotic cell death and the release of inflammatory cytokines of IL-1 family following pyroptosis elicit inflammatory responses in the host (Pasparakis and Vandenabeele, 2015).

1.4.1. Apoptosis

Apoptosis is the most well characterized form of programmed cell death that is involved in the vital processes of the body such as embryonic development and elimination of self-reactive immune cells for homeostasis (Elmore, 2007). In ancient times, Greek texts used the word apoptosis to describe the "falling off" of leaves from the tree, thus reflecting the meaning of apoptosis as a natural physiological process to remove the unwanted components without causing harm to the neighboring parts (Kerr et al., 1972). The term apoptosis was coined in 1972 by Kerr and colleagues describing the typical morphological features associated with this kind of cell death (Kerr et al., 1972). With the advent of new microscopy techniques, different morphological features of apoptotic cell death have been identified such as cell shrinkage, dense cytoplasm, chromatin condensation or pyknosis, plasma membrane blebbing and cellular fragmentation into small apoptotic bodies through a process called "budding" (Elmore, 2007). These apoptotic bodies express 'eat me' signals that are recognized by the tissue resident phagocytes such as macrophages, dendritic cells and neutrophils that are efficient in clearing them through the process called efferocytosis.

The apoptotic bodies have an intact plasma membrane, restricting the release of intracellular components to the outer environment and thus preventing inflammation (Taylor et al., 2008).

Apoptosis involves the proteolytic cascade of caspases, the cysteine-dependent aspartate specific proteases, that execute the final disintegration process of apoptotic cell death (Elmore, 2007; Fink and Cookson, 2005). Caspases exist in the normal cell as inactive zymogens. Once activated, they activate other pro-caspases inducing the proteolytic cascade, leading to cell death. Apoptotic cell death takes place through two distinct pathways, the intrinsic pathway and the extrinsic pathway (Fig. 1) (Fink and Cookson, 2005).

1.4.1.1. Intrinsic apoptosis

The intrinsic apoptotic pathway is also called mitochondrial pathway which is not mediated by ligand receptor binding at the cell surface, but is initiated by various internal factors such as stress, deprivation of growth factors, hormones, radiation, toxins, hypoxia or exposure to free radicals (Fig. 1) (Elmore, 2007). The signals initiated by these factors induce changes in the inner mitochondrial membrane that results in opening of the mitochondrial permeability transition pore (MPT) and consequent loss of mitochondrial transmembrane potential. These events result in the release of various pro-apoptotic mediators from the mitochondria such as cytochrome c, second mitochondria-derived activator of caspases (SMAC/DIABLO) and apoptosis-inducing factor (AIF) (Elmore, 2007). The released cytochrome c binds to the cytosolic protein apoptotic protease-activating factor 1 (APAF-1) in the presence of dATP or ATP. Binding of cytochrome c to the APAF-1 induces oligomerization of APAF-1 molecules which results in the formation of a heptameric wheel like structure, a 1.4 MDa complex called “apoptosome” (Bao and Shi, 2007). APAF-1, being the central component of the apoptosome complex, interacts with initiator caspase, the

pro-caspase 9 through its CARD domain, resulting into the recruitment, cleavage and activation of pro-caspase 9 to catalytically active caspase 9 (Bao and Shi, 2007). The activated caspase 9 interacts with the executioner caspases such as pro-caspase 3, 7 and 6 resulting in their cleavage and activation. These active executioner caspases then cleave various substrates such as PARP, the nuclear protein NuMA and cytokeratins. Further, caspase 3 is also involved in DNA degradation, chromatin condensation and fragmentation of the cells into apoptotic bodies and cleavage of actin filaments resulting into the disintegration of the cytoskeleton of the cell and thus inducing various morphological and biochemical changes associated with the apoptotic cell death (Elmore, 2007).

1.4.1.2. Extrinsic apoptosis

Extrinsic apoptotic pathway is induced by various cell surface receptor-mediated interactions (Fig. 1). The transmembrane receptors involved in the extrinsic apoptotic pathway are members of the TNFR gene superfamily such as TNF-R1, FAS, TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1) and TRAIL-R2. Among these, FASL/FAS- and TNF-α/TNF-R1- mediated apoptotic pathway have been well characterized. These receptors have an extracellular domain for the ligand binding and an 80 amino acids rich cytoplasmic domain, also called death domain (DD), through which they recruit the adaptor proteins and transmit the death signal to the intracellular compartment (Fulda and Debatin, 2006). On ligation of FASL to its cognate receptor FAS, there is a conformation change in the death domain of the receptor, which leads to the recruitment of an adaptor protein FAS-associated death domain (FADD) (Taylor et al., 2008). FADD consists of two domains, a death domain (DD) through which it interacts with the FAS and a death effector domain (DED) through which it interacts with pro-caspase 8 and most probably pro-caspase

10 as well (Peter and Krammer, 2003). This aggregation of FADD and caspase 8 on the cytoplasmic domain of FAS forms a large protein complex named death-inducing signaling complex (DISC). The close proximity of pro-caspase 8 molecules induces their dimerization leading to their auto-proteolytic cleavage and subsequent activation (Bao and Shi, 2007). The active caspase 8, then goes on to cleave and activate the executioner caspases, caspase 3, 6 or 7 leading to apoptotic cell death (Taylor et al., 2008). Interestingly, caspase 3 mediated feedback activation of caspase 8, 9 and 10 has been reported in TNF-α induced apoptosis which indicates the role of caspase 3 as not only the executioner caspase but also important in feedback amplification of the apoptotic loop (Yang et al., 2006).

One of the regulators of apoptotic pathway is a protein called cellular FLICE inhibitory protein (cFLIP). $cFLIP_L$, one of the variants of cFLIP, resembles caspase 8 in structure but lack the catalytic site. $cFLIP_L$ can form heterodimers with caspase 8 that limits the activation of caspase 8 and blocks apoptosis (Wilson et al., 2009). In addition to cFLIP, the members of the BcL-2 family of proteins also control the apoptotic events. Some members of the family are pro-apoptotic proteins such as Bax, Bak, Bid and Bim while some members such as Bcl-2, Bcl-x, Bcl-XL and Bcl-XS are anti-apoptotic and rescue the cell from apoptotic process (Elmore, 2007).

Occasionally, there is a crosstalk between extrinsic and intrinsic pathway through the caspase 8 mediated cleavage of BH3 only protein, BID. After cleavage, the truncated BID (tBID) induces mitochondrial permeability to release cytochrome c and initiate intrinsic or mitochondrial apoptotic pathway and thus aggravates the apoptotic process through both intrinsic and extrinsic pathways (Taylor et al., 2008).

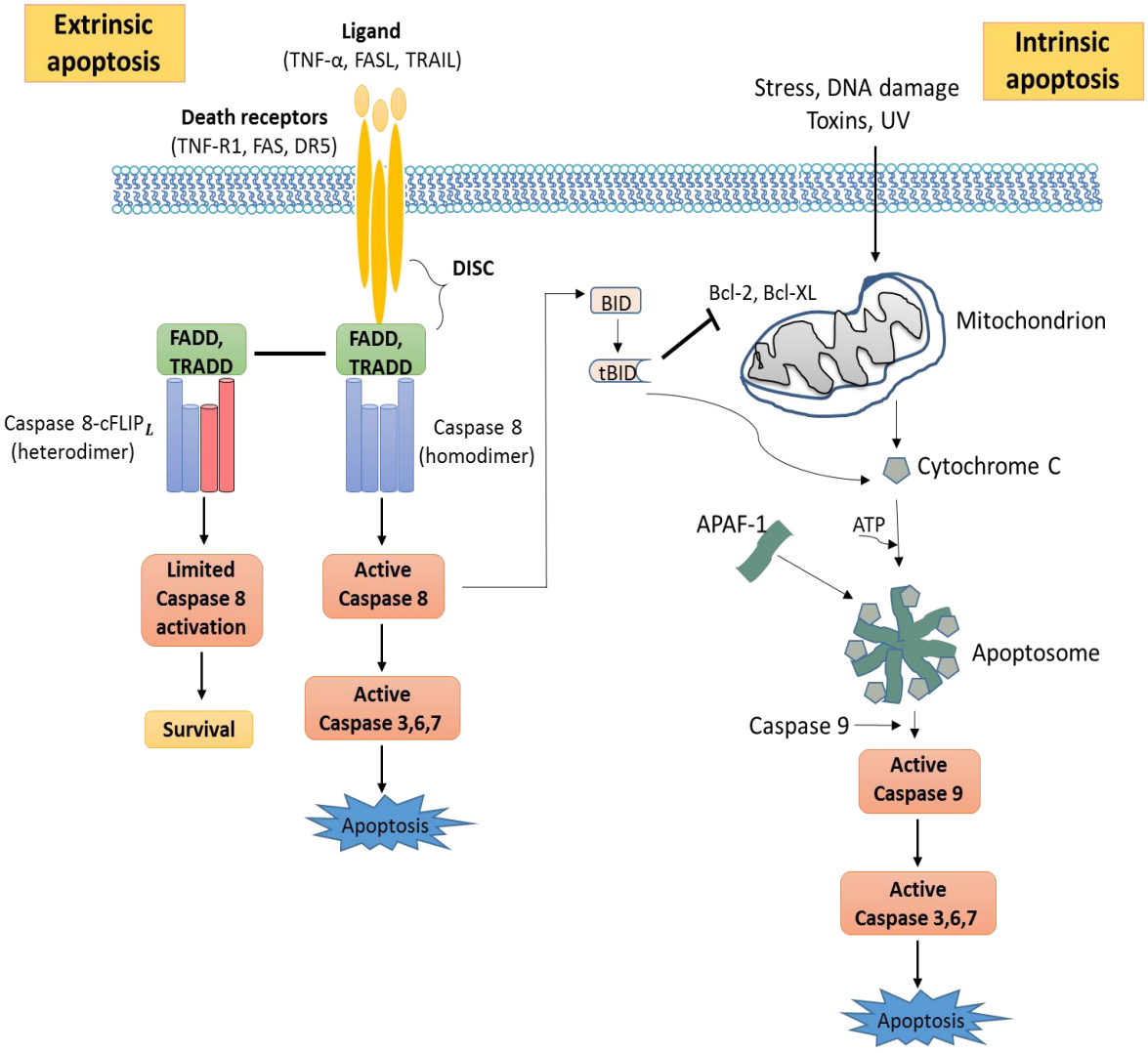

Extrinsic apoptosis
Ligand
(TNF-α, FASL, TRAIL)
Death receptors
(TNF-R1, FAS, DR5)
DISC
FADD, TRADD
FADD, TRADD
Caspase 8-cFLIP$_L$
(heterodimer)
Caspase 8
(homodimer)
Limited Caspase 8 activation
Survival
Active Caspase 8
Active Caspase 3,6,7
Apoptosis
BID
tBID
Bcl-2, Bcl-XL
Intrinsic apoptosis
Stress, DNA damage
Toxins, UV
Mitochondrion
Cytochrome C
APAF-1
ATP
Apoptosome
Caspase 9
Active Caspase 9
Active Caspase 3,6,7
Apoptosis

Figure 1. The extrinsic and intrinsic pathways of apoptosis. Extrinsic apoptosis is induced by various members of the TNFR gene superfamily. On ligation of the ligand with the death receptor, there is recruitment of adaptor protein (TRADD or FADD) forming a death-inducing signaling complex (DISC). DISC formation recruits caspase 8 to form homodimers resulting in their autocatalytic cleavage and activation. Activated caspase 8 then activates executioner caspases, caspase 3, 6 or 7 to induce apoptosis. In case of caspase 8 and $cFLIP_L$ heterodimerization, there is limited activation of caspase 8 that promotes cell survival. Intrinsic apoptosis is triggered by various kind of stress signals resulting in the release of cytochrome c from the mitochondria. Cytochrome c binds to APAF-1 and forms a heptameric structure called apoptosome that activates pro-caspase 9. Activated caspase 9 activates executioner caspases to induce apoptosis. There is a crosstalk between the two pathways through caspase 8 mediated cleavage of BID to form tBID. tBID inhibits anti-apoptotic proteins (BCL-2 and BCL-XL) and induces mitochondrial permeability to release cytochrome c to initiate intrinsic apoptosis.

1.4.2. Necrosis and Secondary necrosis

Necrosis is considered as an accidental, passive and a toxic form of cell death, that occurs in response to various environmental stress factors such as sudden changes in temperature, osmotic pressure or pH, intensive cell membrane damage, depletion of ATP and other energy supplies (Elmore, 2007; Fink and Cookson, 2005; Yang et al., 2015). The basic morphological features of necrosis are cell swelling (also known as oncosis), disintegrated mitochondria and ribosomes, ruptured lysosomes, formation of cytoplasmic vacuoles and blebs and loss of cell membrane integrity (Vanlangenakker et al., 2012). The cell membrane rupture results in the release of the intracellular contents also known as DAMPs in the environment, which triggers inflammation. Various diseases that are associated with sterile inflammation have been correlated with necrotic cell death such as gout, atherosclerosis, and ischemia reperfusion (Yang et al., 2015). Recently various regulated form of necrosis have been identified. Necroptosis, a programmed cell death, has been the most studied and well-characterized form of regulated necrosis till date (Pasparakis and Vandenabeele, 2015).

Following apoptosis, phagocytes rapidly pick up the apoptotic blebs by the process called efferocytosis. In case this process is hampered by any means and phagocytes do not clear up the apoptotic cells, the cells undergo secondary necrosis (Nagata and Tanaka, 2017). The cell rupture following secondary necrosis causes the intracellular contents to be dissipated in the extracellular environment that induces inflammatory conditions (Nagata and Tanaka, 2017). There are arguments regarding secondary necrosis being an artifact of *in vitro* conditions rather than being a physiological process *in vivo*. However, it has been speculated that it may have an important role in the (patho) physiological role in case of extensive apoptosis going on in the tissues (Berghe et al., 2010).

1.4.3. Ripoptosome signaling

Cell death signaling is an intricate process that is initiated on multiprotein complexes. Apoptosome, DISC and inflammasome are the complexes that have been known for many years, however new cell death inducing complexes such as necrosome and ripoptosome have been identified recently (Schilling et al., 2014). In the context of TNF-R1 signaling (Fig. 2), the ligation of TNF-α results in the recruitment of TRADD and other proteins such as RipK1, cIAP1/2 and TRAF2 to form complex I. The K63 linked ubiquitination of RipK1 by cIAPs ensues the participation of RipK1 in complex I, also called the survival complex, which recruits other kinase complexes that leads to the activation of NF-kB (Blander, 2014). Loss or degradation of cIAPs results in disassembly of complex I, and RipK1 then interacts with other proteins such as FADD, caspase 8 and cFLIP that has been described as the ripoptosome complex (Darding and Meier, 2012). The Ripoptosome complex is a large ~ 2-MDa macromolecular cytosolic complex consisting of RipK1, RipK3, FADD, caspase 8 and two isoforms of cFLIP. This complex is dependent on the kinase function of RipK1 and thus is termed "Ripoptosome" (Feoktistova et al., 2011; Tenev et al., 2011). Unlike complex I, ripoptosome is not dependent on ligands such as FASL or TRAIL, and can be spontaneously formed in the cytosol in response to cIAPs depletion (Feoktistova et al., 2011; Tenev et al., 2011). Ripoptosome, itself is not able to induce cell death, but its formation sensitizes the cells to different ligands such as TNF, TWEAK or TLR3 agonist (Poly (I:C) (Darding and Meier, 2011; Schilling et al., 2014). Ripoptosome has been reported to be formed in response to various factors such as DNA damage, genotoxic stress-mediated depletion of IAP proteins, accumulation of RipK1, treatment with SMAC mimetics and etoposide (Schilling et al., 2014; Tenev et al., 2011). Ripotosome was also reported to induce cell death in the context of TLR3 signaling (Feoktistova et al., 2011).

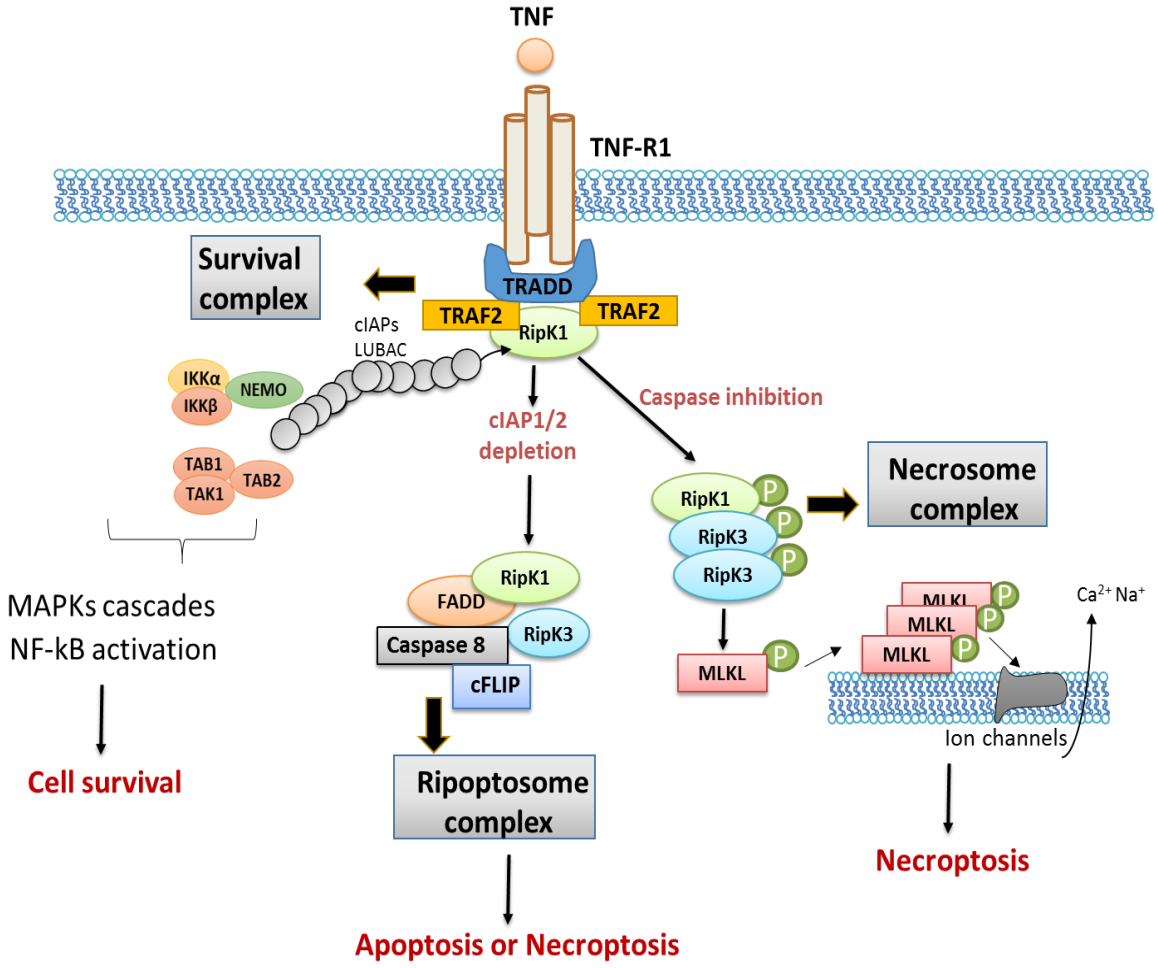

TNF
TNF-R1
Survival complex
TRADD
TRAF2
TRAF2
RipK1
cIAPs
LUBAC
IKKα
IKKβ
NEMO
TAB1
TAK1
TAB2
Caspase inhibition
cIAP1/2 depletion
MAPKs cascades
NF-kB activation
Cell survival
RipK1
FADD
RipK3
Caspase 8
cFLIP
Ripoptosome complex
Apoptosis or Necroptosis
RipK1
RipK3
RipK3
Necrosome complex
MLKL
MLKL
MLKL
MLKL
Ca2+ Na+
Ion channels
Necroptosis

Figure 2. RipK1 participates in three distinct complexes downstream of TNF-R1 signaling. TNF-α binding to its cognate receptor TNF-R1, initiates the signaling cascade where RipK1 play a central role in the formation of different complexes. Ubiquitination of RipK1 by cIAPs and LUBAC ensues participation of RipK1 in the survival complex that leads to the activation of NF-kB pathways and MAPKs cascades. In case of cIAP1/2 depletion, RipK1 dissociates from the complex and interacts with FADD and caspase 8 to form the ripoptosome complex. Ripoptosome complex can induce apoptosis by caspase 8 activation or necroptosis by RipK1 and RipK3 interaction. If caspase 8 is inhibited, the interaction of RipK1 and RipK3 is promoted. Activated RipK3 mediates phosphorylation of MLKL to form necrosome complex that results in necroptosis.

Ripoptosome is at the crossroad between two distinct pathways, apoptosis and necroptosis where the fate of cellular death depends on the activity of caspase 8 and other components of the complex (Schilling et al., 2014). The caspase 8 activity behaves as a rheostat in the ripoptosome complex, the activation of which induces caspase 8 mediated apoptosis and inhibition of which induces RipK1 and RipK3 mediated necroptosis. If caspase 8 activity is inhibited, then a pseudo kinase, mixed lineage kinase domain-like protein (MLKL), interacts with RipK3 as part of the necrosome complex and induces necroptosis (Pasparakis and Vandenabeele, 2015) (Fig. 2).

Ripoptosome-induced cell death has been associated with autocrine TNF-α production in many cancer cell lines (Petersen et al., 2007). cIAP1/ 2, X-linked IAP (XIAP) and $cFLIP_L$ are the negative regulators of ripoptosome complex formation (Darding and Meier, 2012; Tenev et al., 2011).

1.4.3.1. Inhibitors of Apoptosis Proteins (IAPs)

IAPs are the family of proteins that regulate the caspase activity and also control the ubiquitin dependent pathways for the NF-kB activation and other signaling mechanisms in the innate immune response. IAPs comprise of many members among with cIAP1, cIAP2 and XIAP are the well-characterized ones and important in the innate immune response (Imre et al., 2011). cIAPs and XIAP are known to ubiquitinate various components of the ripoptosome complex and thus suppress its formation. Also, the knockdown of all three IAPs at the same is required for a full ripoptosome complex formation (Tenev et al., 2011). Thus, in case of the depletion of IAPs, there is spontaneous formation of the ripoptosome (Dickens et al., 2012). Mammalian cIAPs and XIAP have three BIR (Baculoviral IAP repeat) domains that mediate interactions with different proteins for anti-apoptotic activity, a UBA (ubiquitin-

associated domain) that helps to bind to the ubiquitin chains and a carboxy terminal RING (Really interesting new gene) domain for its E3 ubiquitin ligase activity (Darding and Meier, 2012; Dickens et al., 2012). XIAP is considered as a direct inhibitor of caspase 9, caspase 3 and caspase 7. It binds to the caspases on their IAP-binding motifs (IBM) through its BIR2 and BIR3 domains and inhibit the activity of caspases (Blander, 2014). Unlike XIAP, cIAPs do not directly bind and inhibit caspases but are more involved in ubiquitin mediated activation of the survival pathways such as NF-kB and thus limiting the induction of cell death pathways (Darding and Meier, 2012).

These IAP members are reported to be deregulated and mostly over-expressed in many cancers (Hunter et al., 2007). This results in the inhibition of the apoptotic cell death, leading to chemoresistance and disease progression because of which they have been the targets of various chemotherapies (Tenev et al., 2011). Small pharmacological inhibitors of IAPs have been developed for the better understanding of IAPs and their role in cancers (Darding and Meier, 2012).

1.4.3.2. SMAC/SMAC mimetic

During intrinsic apoptosis, along with cytochrome c there is a release of another protein called second mitochondria-derived activator of caspases (SMAC, also known as DIABLO) from the mitochondria. SMAC binds with the IAP proteins through a highly conserved four-residue (AVPI) IAP-binding motif (IBM) at its amino terminus and renders them inactive by degradation or inhibition. This way SMAC promotes caspase activation and induces apoptotic cell death (Fig. 3) (Blander, 2014). SMAC mimetics (SMs) are the synthetic molecules designed to mimic the N-terminal IAP-binding motif (IBM) of SMAC protein that induces the formation of ripoptosome within minutes of treatment. Most of the

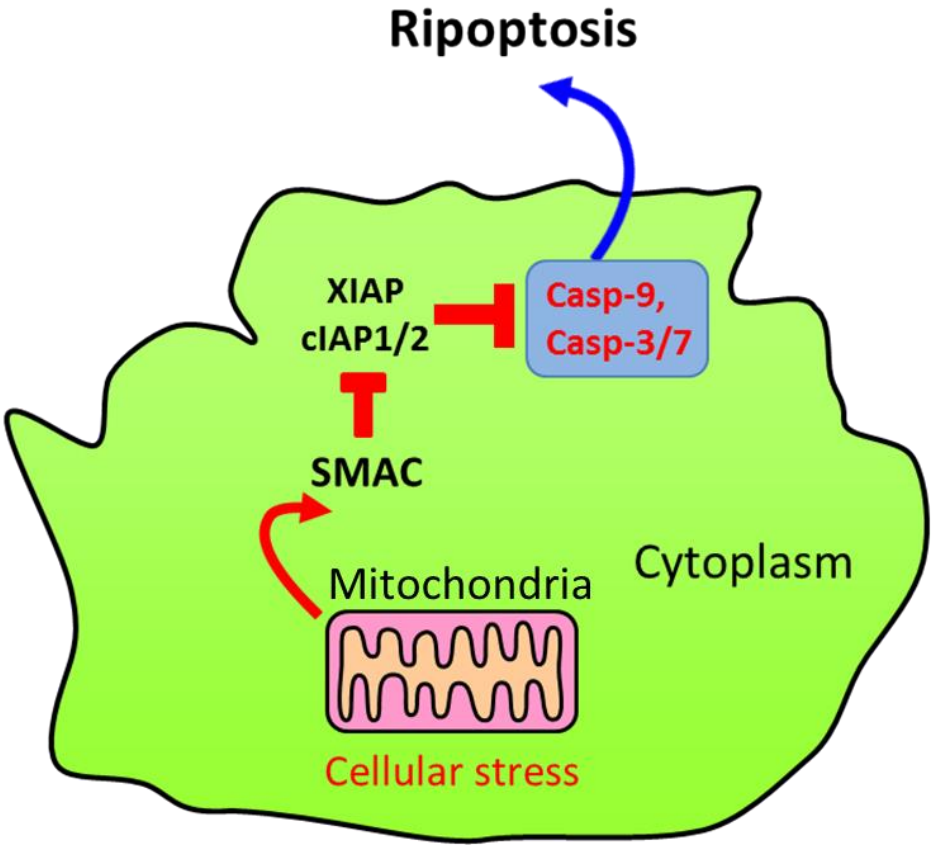

Ripoptosis
XIAP
cIAP1/2
Casp-9,
Casp-3/7
SMAC
Mitochondria
Cytoplasm
Cellular stress

Figure 3. SMAC induces ripoptosome signaling by inhibiting the inhibitors of caspases. In the steady state, the IAP proteins, XIAP and cIAP1/2 inactivate the apoptotic caspases. During intrinsic apoptotic signaling, SMAC protein is released from the mitochondria. The SMAC protein inactivates the IAPS by binding to their BIR domain. This inhibition of the IAPs renders the caspases free from their inhibition and thus gets activated to induce cell death (Ripoptosis-ripoptosome induced cell death).

SMs are developed as dimers, as the dimers of SMAC are active in the physiological process (Wu et al., 2007). SM could be monovalent having one AVPI motif or bivalent having two AVPI motifs. Bivalent SMs are more efficient in binding to IAP proteins, especially to XIAP, leading to better caspase activation (Almagro and Vucic, 2012). SMs can bind to many IAP proteins through their BIR2 or BIR3 domains. SMs are most effective for cIAP1 and cIAP2, where binding of SMs on the BIR3 domain of cIAP1 or cIAP2 results in its conformational change allowing its dimerization. Dimerization of cIAP1/2 activates its E3 ubiquitin ligase activity leading to their auto-ubiquitination and proteasome mediated degradation within minutes of SM binding (Almagro and Vucic, 2012). cIAPs are known to positively regulate canonical NF-kB and negatively regulate non-canonical NF-kB activation (Tenev et al., 2011). However, the degradation of cIAPs induces both the canonical and non-canonical NF-kB activation resulting in TNF-α secretion. The mechanism of canonical NF-kB activation is not very clear though. The TNF-α produced then activates caspase 8 mediating apoptotic cell death (Wu et al., 2007). SMs can directly induce degradation of cIAP1, however degradation of cIAP2 depends on the presence of cIAP1 (Darding and Meier, 2012). SMs bind to XIAP thorough its IBM and thus inhibit its binding and inactivation of caspase 3, 7 and 9 (Almagro and Vucic, 2012). SMs are reported to induce cell death as a single agent in many cancer cell lines, however some cancer cells are sensitive to the combination of SM and exogenous TNF-α and some are totally resistant to SM indicating the absence or inactive ripoptosome formation in those cells (Wu et al., 2007). Many SMs are in different phases of clinical trials. SMs offer a potential therapeutic for many cancers as a single agent or in combination with other chemotherapies or radiation therapies (Petersen et al., 2007).

One of the important members of the ripoptosome complex is cFLIP, which regulates caspase 8 activity and modulates the signaling outcome in the ripoptosome complex (Dickens et al., 2012). cFLIP has 11 isoforms among which two isoforms $cFLIP_L$ and $cFLIP_S$ are highly expressed in the cells. $cFLIP_L$ resembles caspase 8 in structure but lacks the catalytic enzyme activity. Thus $cFLIP_L$ can bind to caspase 8 to form a heterodimer. Unlike the homodimer of caspase 8 molecules which has higher proteolytic activity, the caspase 8 and $cFLIP_L$ heterodimer has limited proteolytic activity that can still cleave RipK1 and RipK3 but cannot cleave apoptotic substrates and thus is unable to induce apoptosis (Darding and Meier, 2012; Schilling et al., 2014). By inactivating RipK1 and RipK3, $cFLIP_L$ protects cells against both apoptosis and necroptosis. On the other hand $cFLIP_S$ consist of only the pro-domain that can bind to FADD and heterodimerize with caspase 8 which results in complete inhibition of caspase 8 activation. The inactive caspase 8 is now ineffective in cleaving both Rip kinases and also in inducing apoptotic cell death (Darding and Meier, 2012). RipK1 can now interact with RipK3 and thus initiate necroptotic pathway. Thus $cFLIP_S$ inhibits apoptosis but promotes necroptosis in the cells (Schilling et al., 2014).

The discovery of ripoptosome has provided new insights for the treatment of cancer cells by the induction of caspase independent cell death in apoptotic resistant cancer cells. Understanding the mechanisms of ripoptosome signaling and its various switch points would be helpful for many therapeutics associated with cancers and other diseases associated with cell death resistance.

1.4.4. Necrosome signaling

Necrosis was considered as an accidental form of cell death for many years (Fuchs and Steller, 2015). The new genetic and functional evidences have revealed that in certain conditions necrosis can be induced through a highly regulated pathway that is known as necroptosis (Pasparakis and Vandenabeele, 2015). Many stimuli can trigger necroptosis such as TNF-α, IFN-β, DNA damage, SMAC mimetic treatment, viral infection and ligands of TLR-4, TLR-3, but this pathway is induced only when caspase activation is suppressed (Chan et al., 2015; Tait et al., 2014; Tenev et al., 2011). TNF-α/TNF-R1 signaling mediated necroptosis is the best-characterized pathway till date. RipK1 and RipK3 are the important members of Ser/Thr kinase family that have essential role in immune signaling. RipK1 contains an amino-terminal kinase domain that is important for phosphorylating various substrates, an intermediate RIP homotypic interaction motif (RHIM) domain and a carboxy-terminal death domain (Ofengeim and Yuan, 2013). Both RipK1 and RipK3 contain the RHIM domain, which is important for interaction with other RHIM containing proteins. When caspase activation is suppressed, RipK1 interacts with RipK3 to form a large amyloid-like filamentous complex called the necrosome (Tait et al., 2014). Programming for necrosome signaling starts with the phosphorylation of RipK1, which enables it to interact with RipK3 through RHIM domain and to induce the phosphorylation of RipK3. Activation of RipK3 induces its oligomerization and consequent phosphorylation of its substrate Mixed-lineage kinase domain-like (MLKL). Phosphorylation of MLKL induces its oligomerization and translocation to the plasma membrane where it induces membrane lysis through the interaction with calcium and sodium ion channels, inducing influx of cations or by direct binding to the membrane lipids and disintegrating the membrane (Chan et al., 2015). MLKL

was identified as a downstream substrate of RipK3 in the execution of necroptosis as the genetic deletion of MLKL was reported to impart complete resistance to necroptosis (Wu et al., 2013). Since the kinase activity of RipK1 is important for the activation of RipK3 and necrosome signaling, a RipK1 kinase inhibitor, necrostatin-1, was developed in 2005 by Yuan group, which helped researchers delineate necroptosis signaling *in vivo* and *in vitro* (Degterev et al., 2005). RipK3 is reported to be indispensable for necroptosis while RipK1 is dispensable in some cases such as TRIF- and DAI-mediated necroptosis (Chan et al., 2015; Upton and Chan, 2014). Downstream of TLRs, the TRIF protein has RHIM domain through which they can directly interact with RipK3 and initiate necroptosis (Tait et al., 2014).

The membrane lysis following necroptosis releases large number of DAMPs into the extracellular milieu. These DAMPs act as triggers of inflammation. The inflammation in response to necroptotic cell death is beneficial in case of early immune response against pathogens, however the massive release of DAMPs is associated with various inflammatory pathologies such as Atherosclerosis (Lin et al., 2013), Myocardial infarction (Smith et al., 2007), Psoriasis (Bonnet et al., 2011; Kovalenko et al., 2009) and Ischemia-reperfusion kidney injury (Linkermann et al., 2013). Many viruses are known to express caspase inhibitors such as protein CrmA by coxpox virus and vFLIP proteins by herpes virus that inhibits the apoptotic cell death in the cells. In such cases necroptosis acts as a backup mechanism to induce cell death in virally infected cells and impart protection to the host (Tait et al., 2014). However, MCMV virus has been reported to encode apoptosis inhibitor, vICA as well as necroptosis inhibitor vIRA indicating the importance of necroptosis in host pathogen interactions and the evolution of the microbes against this form of cell death (Chan et al., 2015).

2.0 HYPOTHESIS AND STATEMENT OF OBJECTIVES

2.1. Rationale

Recently, several alternative forms of cell death have been reported (Yang et al., 2015), and some of these cell death pathways share a few characteristics with apoptosis. Recent work has shown that during infection, cell death of immune cells operates through these alternative pathways of cell death. Since myeloid cells play a key front line role in controlling infections early on, the induction and regulation of cell death of myeloid cells through these alternative cell death pathways is not clear. Furthermore, monocytes, which are one of the dominant myeloid cell types in the peripheral blood, differentiate into macrophages, and the relative impact of this differentiation program on their susceptibility to cell death by the alternative mechanisms is not clear. During in vitro differentiation of macrophages from bone marrow cells, I observed significant differences between macrophages isolated at day 5 and day 12 of growth in terms of morphology and their response to cell death. Macrophages isolated at days beyond day 12 behaved in a similar manner as day 12 macrophages. Thus, in my thesis I have investigated the mechanisms behind the differences in day 5 and day 12 macrophages in terms of cell death.

2.2. Hypothesis

Since macrophages thrive in harsh and toxic environments and persist for prolonged periods, we hypothesize that during the differentiation of monocytes to macrophages, there must be programming towards reduced cell death that is mediated by mechanisms that regulate the activation of key caspases.

2.3. Objectives

1. Evaluate the impact of macrophage differentiation on ripoptosome signaling
    - Impact on cell viability (MTT assay, Fluorescent microscopy)
    - Role of transcription factors, cytokine receptors and Rip kinases
    - Role of caspases
    - Impact on key protein interactions in ripoptosome signaling
2. Impact of TLR activation on ripoptosome signaling
    - Impact on cell viability
    - Role of transcription factors, cytokine receptors and Rip kinases
    - Role of caspases
    - Role of inhibitors of apoptosis
3. Impact of macrophage differentiation on necrosome signaling
    - Impact of SMAC mimetic-driven necrosome activation
    - Impact of TLR and cytokine-driven necrosome activation

3.0. MATERIAL AND METHODS

3.1. Animals: Mice used in the experiments (listed in table 1) were housed under specific pathogen-free conditions and maintained in accordance with CCAC guidelines at the University of Ottawa Animal facility. Protocols and procedures were approved and monitored by the University of Ottawa Animal Care Committee and Ethics Board. All mice were age and sex-matched for experiments. Unless stated otherwise, all knockout mice were on a C57BL/6 genetic background.

3.2. Generation of Bone Marrow Derived Macrophages: Murine bone marrow derived macrophages were used in the experiments. The protocol includes the following steps: Mice were euthanized with CO2 exposure and 70% alcohol was sprayed over the body to decontaminate the surface before incision. With the use of scissors and tweezers, the fur surrounding the fore and hind limbs were removed and the limbs were excised. Muscles and tissue were carefully removed and the bones after thoroughly being cleaned with 70% ethanol was placed in RPMI+8% FBS + 55 μM 1000X β- mercaptoethanol (2ME) + 50 ng/ml gentamycin. To harvest pluripotent hematopoietic stem cells, a small gauge needle was used to flush bones with R8+gentamycin. Bone marrow was flushed through a 100 μm cell strainer into a 50 ml falcon tube. A small 10 μl aliquot was taken to count the number of cells while remaining cells were centrifuged at 1600 RPM for 8 minutes. 50 mm plastic Petri dishes were coated with macrophage colony stimulating factor (M-CSF) at the final concentration of 5 ng/ml. To prevent macrophages from clumping in culture, M-CSF was spread evenly over the bottom of the petri dish using an L shaped spreader. Following centrifugation, the cell pellet was resuspended in R8+gentamycin and approximately $15x10^6$

cells were added to each petri dish. R8 + gentamycin was added to Petri dishes for a final volume of 10 ml. The Petri dishes were incubated at 37°C to allow macrophage differentiation. The purity of macrophages was confirmed by flow cytometry (CD11b+/F4/80+/Ly6C-).

Table 1. Mouse strains used for experiments

Mouse Strain	Source	Other Information
C57BL/6	Jackson Laboratories	Stock #000664
TNF-R1/2-/-	Jackson Laboratories	Stock #003243
TNF-R1-/-	Jackson Laboratories	Stock #003242
TNF-R2-/-	Jackson Laboratories	Stock #002620
TNF-α-/-	Jackson Laboratories	Stock #005540
IFNAR1-/-	A kind gift from Dr. Kaja Murali-Krishna (Emory University, USA)	Mice were backcrossed to C57BL/6 for 14 generations as described by Kolumam et al. (2005) from mice generated as described by Müller et al. (1994)
TRIF-/-	Jackson Laboratories	Stock #005037
MYD88-/-	Jackson Laboratories	Stock #009088
XIAP-/-	A kind gift from Dr. Robert Korneluk University of Ottawa	Generated as described by Harlin et al. (2001)
RipK1^{K45A}	A kind gift from Dr. Peter J. Gough (GlaxoSmithKline, Collegeville, USA)	Generated as described by Berger et al. (2014)
RIPK3-/-	A kind gift from Dr. Vishva Dixit (Genentech, USA)	Generated as described by Newton et al. (2004)
MK2-/-	A kind gift from Dr. Matthias Gaestel Hannover Medical School Germany	Generated as described by Kotlyarov et al. (1999)

3.3. Purification of Monocytes: Bone marrow cells were harvested in the similar manner and was flushed through a 70 μm cell strainer into a 50 ml falcon tube using R8 media. RBC lysis buffer was used to lyse RBCs. A small aliquot (10μl) was taken to count the number of cells while remaining cells were centrifuged at 500xg for 5 minutes. The supernatant was discarded and the cells were resuspended at $1x10^8$ cells/ml in 1% FBS PBS media. EasySep Mouse Monocyte Enrichment Kit (Stemcell Technologies, Catalog #19861) was used to purify and isolate monocytes. The purity of monocytes was confirmed by flow cytometry (CD11b+ /Ly6C+/ Ly6G-).

3.4. *In vitro* Inhibitor Assays: Bone marrow derived macrophages (BMDMs) at day 5 and day 12 of growth with M-CSF, were plated in 96 well flat bottomed plate (Falcon). Around 7×10^4 cells were seeded per well and incubated overnight to ensure adherence and also to avoid stressed state of the cells due to scraping and plating conditions. In case of monocytes, the isolated monocytes were seeded in 96 well flat bottomed plate and incubated for 1-2 hours before treatment. Many different small molecule inhibitors and agonists (listed in table 2) were used to treat the macrophages and monocytes. The details of each treatment will be provided in the text. Following treatment with appropriate concentrations of inhibitors and agonists, cells were usually left for 24 hours before cell death or viability was measured.

Table 2. Inhibitors and agonists used for experiments

Inhibitor/Agonist	Source	Notes	Concentrations
Birinapant (BP)	Selleckchem (Cat. #S7015)	SMAC mimetic	1-20 μM
BV6	Obtained from Genentech, USA	SMAC mimetic	10 μM
Necrostatin-1 (Nec-1)	Sigma-Aldrich (Cat. # 9037)	Inhibitor of RIPK1 kinase activity	10 μM
Z-VAD-FMK	ApexBio (Cat. # A1902)	Pan-caspase inhibitor	10-50 μM
Z-IETD-FMK	ApexBio (Cat. #B3232)	Inhibitor of caspase-8	50 μM
Z-LEHD-FMK	R &D Systems (Cat. #FMK008)	Inhibitor of caspase-9	50 μM
Z-DEVD-FMK	R &D Systems (Cat. #FMK004)	Inhibitor of caspase-3	50 μM
Ultra-pure LPS from E. coli 055:B5	Sigma-Aldrich (Cat. # L4524)	Cell wall component of gram-negative bacteria used to stimulate TLR4	1 ng/ml
Gsk 872	Glixx (Cat. # GLXC-03990)	Inhibitor of RIPK3 kinase activity	5 μM
LY2228820	Selleckchem (Cat. #S1494)	Inhibitor of p38MAPK	5 μM
Recombinant mouse TNF-α	R &D Systems (Cat. #410-MT)	TNFR agonist	1000 U/ml
Recombinant mouse IFN-β	PBL Interferon source (Cat. #12400-1)	IFNAR agonist	1000 U/ml
PDO325901	Selleckchem (Cat. #S1036)	Inhibitor of ERK1/2	20 nM
SP600125	Sigma-Aldrich (Cat. #S5567)	Inhibitor of JNK	10-40 μM

Note: All inhibitors and agonist were diluted in R8+Gentamycin from stock.

3.5. Cell viability test (MTT assay): Cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. A 5% MTT solution (Sigma-Aldrich #M5655) was prepared in R8 medium and 100μl was added to the cells. The

cells were then incubated at 37°C for 2 hours. After 2 hours, the cells were lysed by adding 100µl of a solution of 37% HCL diluted in isopropanol. The solution was mixed vigorously to break the formazen crystals formed by the live cells in response to MTT reagent.

The absorbance of the solubilized dye was measured at 570 nm with a reference wavelength of 650 nm using a FilterMax^{TM} F5 plate reader (Molecular Devices). The absorbance values were normalized to their respective controls (BP treated cells were normalized to untreated cells, LPS+BP and LPS+zVAD to LPS treated cells, TNF-α+zVAD to TNF-α treated cells and IFN-β+zVAD to IFN-β treated cells).

3.6. Immunofluorescence: BMDMS isolated at day 5 and day 12 of growth were treated with reagents as described in the text for 24 hours. Then, the media was aspirated from the well and 100 µl of the staining solution was added to each well. The staining solution includes RPMI lacking phenol with Hoechst (2.5 µg/ml; Invitrogen #33342), a dye for staining dead and live cells and propidium iodide (PI) (1:10 dilution; BD Pharmingen, #550825), a dye for staining dead cells only. The cells were stained for 25-30 minutes and then examined by immunofluorescence microscopy. The live cells are detectable by their blue color and dead cells by their pink (Blue +Red) color. A Zeiss AxioObserver D1 microscope and the AxioVision Rel. 4.8 program were used to capture and analyze images. The Lumenera Infinity Analyze program was used to quantitate the rate of cell death.

3.7. Western blotting: BMDMs after day 5 and day 12 of growth with M-CSF were seeded in a 24-well plate at 3×10^5 cells/well and incubated overnight at 37°C. Next day the cells were treated at different time points with inhibitors or agonists. After the treatment for certain time points, the cells were washed with cold PBS and then lysed with cold 1% SDS

lysis buffer with 1% β-mercaptoethanol. Samples were boiled immediately to minimize protein degradation and were frozen for future use. Standard protocol for western blot analysis was followed. Lysates were run on a 1.5 mm 15-well 8%, 10%, 12% or 15% polyacrylamide gel depending on the size of the proteins of interest. Gels were typically loaded with 20 µl of lysate and run at 130 V for one hour and 15 minutes. A PVDF membrane was soaked in 100% methanol and washed with the transfer buffer, then the transfer was run at 100V for an hour. The transfer was done on ice to prevent overheating. The membrane after transfer was blocked in 5% skim milk in Tris Buffered Saline Solution (TBS – 0.5M Tris, 1.5 NaCl pH 7) with 0.5% Tween- 20 (TBST) or 5% bovine serum albumin (BSA) depending on the manufacturer's protocol for one hour at room temperature on the shaker. Primary antibodies diluted in appropriate blocking buffer (5% milk or BSA), added to the membrane in a sealed plastic pouch and incubated overnight on the shaker at 4°C. Antibodies and corresponding dilutions used are listed in table 3. Membranes were then washed with TBST three times at ten minutes intervals before adding secondary antibody diluted in the appropriate blocking buffer (5% milk or BSA) for 45 min to one hour. Secondary antibody was discarded and membranes were washed with the same interval/wash. Membranes were visualized using either luminol ECL substrate (Thermo Scientific #32106) or the highly sensitive West Femto ECL substrate (Thermo Scientific #34095) depending on the level of protein expression. Photosensitive film (Sigma-Aldrich, Carestream Health #785019) was used to examine protein expression. Results were quantified using densitometric analysis. The densitometric quantification of western blot signals was performed using ImageJ 1.48 software (Maryland, USA).

Table 3. Primary and secondary antibodies used for western blot analysis.

Antibody	Source	Dilution Used
Mouse anti-Rip1	BD (Cat. #610459)	1:1000 in Milk
Rabbit anti-Rip3	ProSci Inc. (Cat. #2283)	1:1000 in Milk
Mouse anti-β-actin	BD (Cat. #612656)	1:1000 in Milk/or BSA
Rabbit anti-cIAP1/2	Cyclex (Cat. #CY-P1041)	1:1000 in Milk
Rabbit anti-p38 MAPK	Cell signaling (Cat. #8690)	1:1000 in BSA
Rabbit anti-Phospho-p38 MAPK	Cell signaling (Cat. #4511)	1:1000 in BSA
Rabbit anti-NF-κB p65	Cell signaling (Cat. #8242)	1:1000 in BSA
Rabbit anti-Phospho-NF-κB p65	Cell signaling (Cat. #3033)	1:1000 in BSA
Mouse anti-FADD monoclonal antibody (1F7)	ENZO (Cat. #ADI-AAM-212-E)	1:1000 in Milk
Rat anti-caspase8	ENZO (Cat. #1G12)	1:1000 in Milk
Rabbit anti-XIAP	Cell signaling (Cat. #2042)	1:1000 in Milk/or BSA
Rabbit anti-cFLIP	Santa-Cruz (Cat. #sc-8347)	1:1000 in BSA
Rabbit anti-Phospho-P100	Cell signaling (Cat. #4810)	1:1000 in BSA
Rabbit anti-Phospho-MK2	Cell signaling (Cat. #3007)	1:1000 in BSA
Rabbit anti-MK2	Cell signaling (Cat. #3042)	1:1000 in BSA
Rabbit anti-Phospho- p44/42 MAPK (Erk1/2)	Cell signaling (Cat. #4370)	1:1000 in BSA
Rabbit anti-p44/42 MAPK (Erk1/2)	Cell signaling (Cat. #4695)	1:1000 in BSA
Rabbit anti-Phospho-SAPK/JNK	Cell signaling (Cat. #4668)	1:1000 in BSA
Rabbit anti-SAPK/JNK	Cell signaling (Cat. #9252)	1:1000 in BSA

3.8. Flow Cytometry: The monocytes and day 5 and day 12 BMDMs were analyzed by flow cytometry. The monocytes obtained after purification or the BMDMs isolated from the petri dish were filtered through a 70 μM cell strainer (Fisher Scientific #22363548). The cells were washed with staining buffer (1% Bovine serum albumin in PBS). FcBlock (anti-CD16/32) (BD Biosciences #553142) was added to the cells in the staining buffer and incubated for 10 minutes at 4^{o}C to prevent the non-specific binding of antibodies. Then, using various flurophore-conjugated antibodies cell surface staining was performed in the staining buffer. Cells were protected from the light and incubated with the antibodies for 30 minutes at 4^{o}C. After that, cells were washed with the staining buffer and re-suspended in flow fixative (PBS containing 1% paraformaldehyde and 0.02% sodium azide). Samples were then acquired on a CyAN-ADP analyzer (Beckman Coulter) and analyzed using Kaluza software (Beckman Coulter, version 1.2).

Table 4: Flow cytometry staining antibodies

Antibody	Conjugated fluorophore	Source	Catalog number
Ly6C	PE	eBioscience	12-5932-82
CD11b	PE-Cy7	eBioscience	2500112-81
Ly6G	FITC	eBioscience	11-5931-82
F4/80	APC-Cy7	eBioscience	47-4801-80

3.9. Caspase Bioassay: The caspase 8, caspase 9 and caspase 3/7 activities were measured by using the Caspase-Glo 8 (Promega, Catalog #G8200), Caspase-Glo 9 (Promega, Catalog #G8210) and Caspase-Glo 3/7 (Promega, Catalog #G8090) luminescent kits. Approximately 7×10^{4} macrophages were seeded per well in 96-well plate and incubated overnight. Next day,

they were treated with different inhibitors or stimulators for 3-6 hours. The kit consists of Caspase-Glo substrate that contains the tetrapeptide sequence specific to each caspases and Caspase-Glo buffer (some kit also consist of proteasome inhibitor). The substrate was mixed with the buffer and the mix was added to the cells in the 96 well plates. The volume of reagent should be equal to the volume of the media on the cells. Then the reagent was mixed well with the sample by pipetting up and down and incubated at room temperature for 30 minutes to 1 hour. The reagent causes lysis of the cells followed by cleavage of the substrate by the active caspase and generation of the luminescence signal. The luminescence generated is directly proportional to the amount of caspase activity present. The luminescence generated in the wells was measured using a FilterMax^{TM} F5 plate reader (Molecular Devices). The relative luminescence unit (RLU) was measured and plotted on graphs using Graphpad prism.

3.10. Immunoprecipitation: Cell lysates were immunoprecipitated with Dynabeads co-immunoprecipitation kit (Invitrogen, Life Technologies, Catalog #14321D, Burlington, Canada) with rat anti-caspase 8 (ENZO, Catolog. #1G12). The caspase 8 antibody was coupled with the dynabeads with incubation at 37^{o}C for overnight and then washing with various buffers. Next day, BMDMs were treated with Birinapant for 4 hours. Then, the cells were washed with ice-cold 1xPBS and lysed in IP lysis buffer. The lysates were sonicated for 10 seconds and then centrifuged at 5000xg for 5 minutes at 4°C to remove large debris and nuclei. 10µl of the supernatant was stored as input and the remaining clarified lysate were then incubated with antibody-coupled dynabeads for 16-24 hours at 4°C on a rocker platform. The unbound fractions were saved at -20°C and the bound proteins were eluted.

10µl of the 4XSDS-loading dye was added to the eluent and boiled for 5 mins @95^{o}C. The final eluent was analyzed by western blot standard protocol.

3.11. TNF-α Bioassay: TNF-α bioassay performed is based on the cytotoxicity of the TNF-α towards the WEHI cell line, a murine fibrosarcoma cell line. It detects the biologically active TNF-α in the samples. 50µl of the RPMI media was added to the 96 well plates. After the treatment of macrophages for 24 hours with stimulators or inhibitors, as indicated in the figures, 50µl of the supernatant was taken and added to the well with media to make the final volume of 100µl in each well. After that, serial dilutions of the samples (each in triplicates) were made by taking out 50µl from each well and adding to the next well. Serial dilutions of the samples were made to examine the TNF-α activity at various dilutions. A serial dilution of the standard with known TNF-α activity (Recombinant mouse TNF-α, R&D Systems, Cat. #410-MT) was also prepared in one row of the plate. After that, WEHI cells were suspended in RPMI media and added to all the wells at 50µl per well to make the final volume of each well to be 100µl and the final number of the WEHI cells to be 5000 per well. After 24 hours incubation, the viability of cells were measured by MTT assay on the plate reader and the results were analyzed by Graphpad prism software. The cell death is directly proportional to the amount of TNF-α activity present in the sample. 1 Unit equals to 50% death of WEHI cells (1 Unit is defined as the concentration of TNF-α required to kill 50% of WEHI cells).

3.12. Statistics: All error bars show standard error of the mean. Unpaired two-tailed student's t-test was used to determine statistical significance. All statistical analysis were performed using Graphpad Prism software.

4.0. RESULTS

4.1. Differentiation of macrophages induces resistance to ripoptosome-induced cell death

4.1.1. Differentiation of macrophages with M-CSF

Bone marrow contains monocytes as well as various precursor cells that can differentiate into various lineages. We wanted to evaluate whether the response of monocytes and differentiated macrophages to various cell death signaling platforms is different. To this end, we purified monocytes, and differentiated bone marrow precursors with M-CSF for 5 days or 12 days *in vitro*. We characterized the populations of cells by measuring the expression of various cell surface molecules that discriminate between monocytes and macrophages. Monocytes were purified from the bone marrow cells using EasySep™ Mouse Monocyte Enrichment Kit. Purified monocytes and differentiated macrophages were stained with antibodies against Ly6C, F4/80, Ly6G and CD11b and assessed by flow cytometry. As expected, monocytes were Ly6C+, F4/80-, Ly6G- and CD11b+ (Fig. 4A). On the other hand, macrophages were Ly6C-, F4/80+, Ly6G- and CD11b+ (Fig. 4A). Furthermore, there was no difference in the expression of F4/80 cell surface marker in macrophages isolated at day 5 (D5) or day 12 (D12) of differentiation with M-CSF, while the expression of CD11b continued to increase after day 5 of differentiation (Fig. 4A). The increased CD11b expression was also observed by increased MFI expression of CD11b in D12 macrophages compared to D5 macrophages and monocytes (Fig. 4B). We wished to evaluate whether differentiation of macrophages for 5 and 12 days with M-CSF results in any impact on the overall recovery of cells. Bone marrow cells were treated with M-CSF and on day 5 and day 12 post-differentiation, cells were isolated from the petri dish and counted using haemocytometer. Our results indicate

that prolonged differentiation of macrophages for 12 days with M-CSF leads to a slightly higher number of macrophages (Fig. 4C). Overall, these results indicate that prolonged differentiation of macrophages with M-CSF does not have any negative impact on macrophage differentiation in terms of cell recovery or the cell surface phenotype of macrophages.

4.1.2. Prolonged differentiation of macrophages results in resistance to cell death by the SMAC mimetic

We wished to determine whether prolonged differentiation of macrophages has any impact on their susceptibility to cell death pathways. When mitochondrial integrity is compromised, second mitochondrial activator of caspases (SMAC) is released, which causes degradation of inhibitors of apoptosis proteins (IAPs) resulting in cell death (Du et al., 2000; Verhagen et al., 2008). SMAC mimetic (SM) developed to mimic the SMAC protein has been reported to induce spontaneous formation of a cell death inducing complex- "Ripoptosome" in the intracellular compartment of the cell and the cell death induced via ripoptosome is dependent on receptor interacting protein kinase RipK1 (Feoktistova et al., 2011; Tenev et al., 2011). Various SMs have been developed for cancer therapy applications (Chen and Huerta, 2009). Initially, we evaluated the susceptibility of monocytes and macrophages to cell death by the ripoptosome pathway. We used the clinical SM, Birinapant (BP). MTT assay was used to measure the cell viability at 24h post-treatment. Live cells are able to form formazan crystals that can be quantitated by absorbance upon dissolution. Treatment of monocytes with BP resulted in significant cell death of monocytes, which was inhibited by co-treatment of cells with the RipK1 inhibitor, Necrostatin-1 (Nec-1) (Fig. 5A). Similarly, D5 macrophages were highly susceptible to cell death by BP treatment (Fig. 5B).

Surprisingly, D12 macrophages displayed significant resistance to BP induced cell death (Fig. 5B). We also evaluated cell death at 6h post BP treatment and observed that cell death was not significant at this time interval, suggesting that cell death induced by BP treatment operates at a slower speed (Fig. 5C). The cell death of D5 and D12 macrophages with BP was further analysed by immunofluorescence microscopy by staining the cells with propidium iodide (PI) (red) and Hoechst (blue). Internalisation of PI is indicative of cell death by necrosis or secondary necrosis in case of apoptotic cells in vitro. D5 macrophages showed positive staining with PI indicating cell death which was not seen in cells treated with Nec-1 along with BP (Fig. 5D). However, D12 macrophages were not stained with PI indicating the cells were live (Fig. 5D). We tested cell death of macrophages following treatment with a different SM, BV6, and observed that D12 macrophages display significant resistance to cell death (Appendix-Fig. I).

Thus, these results indicate that the cell death induced by SMAC mimetic proceeds slowly, and that the prolonged differentiation of macrophages results in acquisition of resistance to this pathway of cell death.

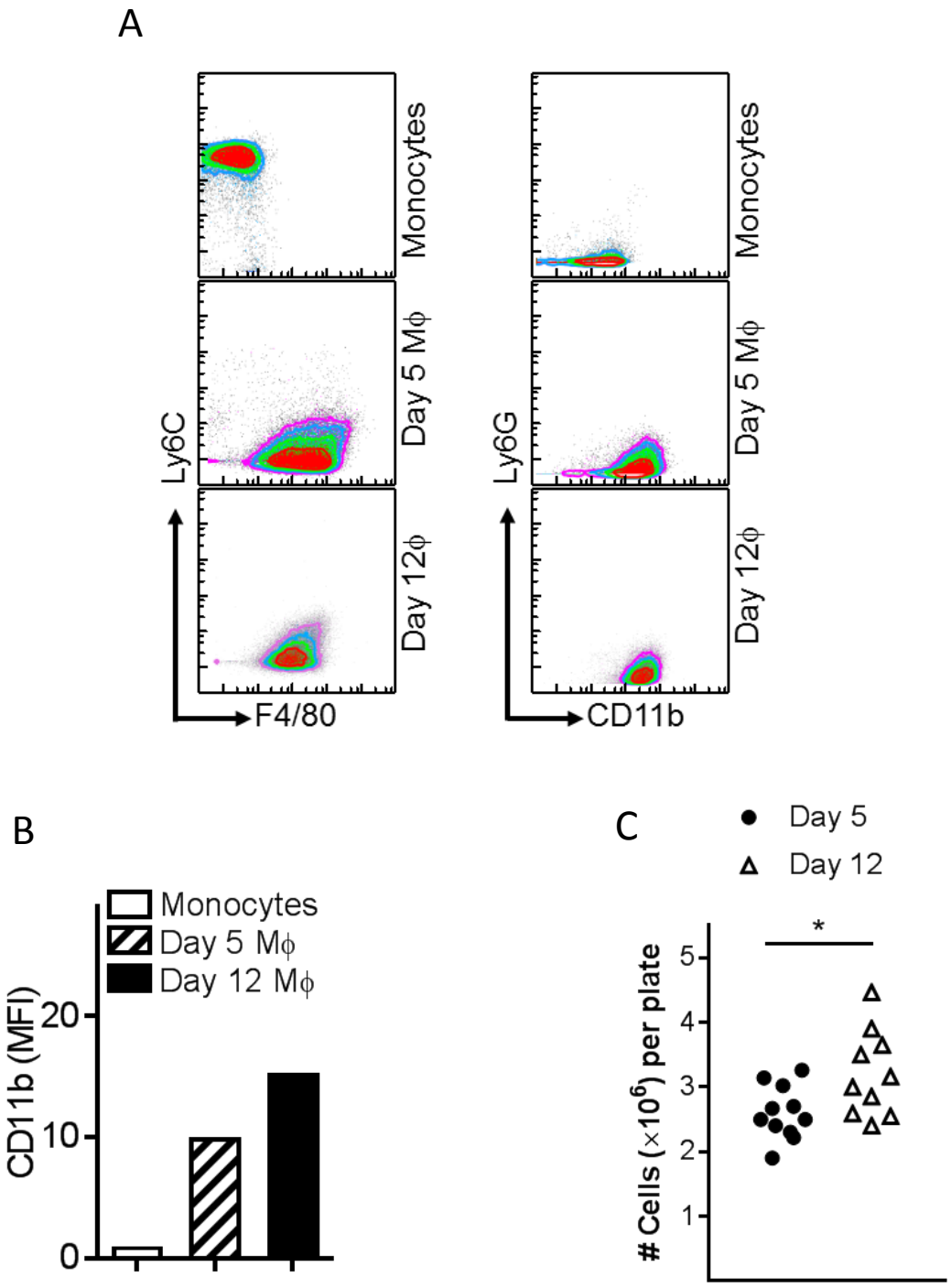
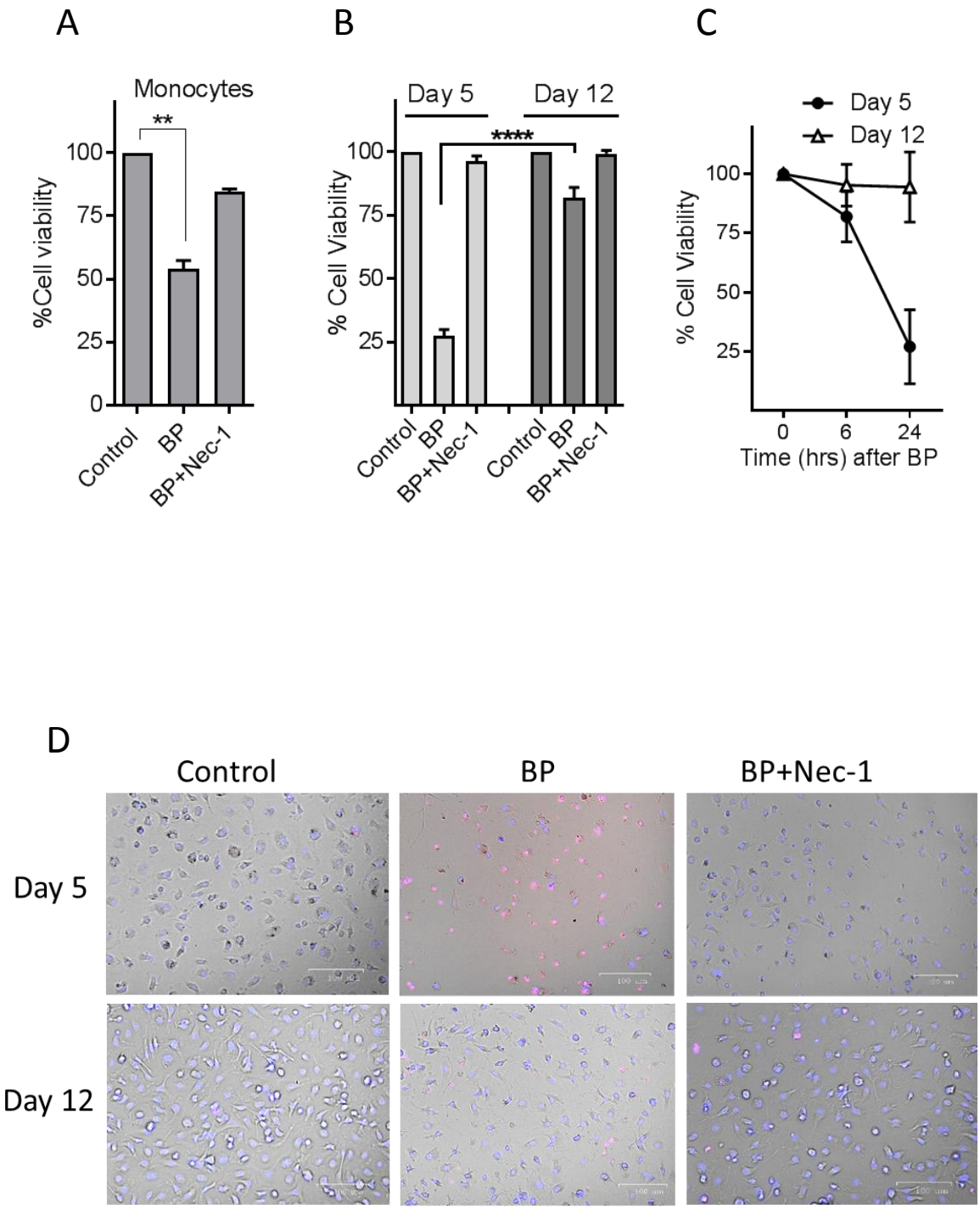

A
Monocytes
Day 5 Mϕ
Day 12ϕ
Ly6C
F4/80
Ly6G
CD11b
B
Monocytes
Day 5 Mϕ
Day 12 Mϕ
CD11b (MFI)
0
10
20
C
Day 5
Day 12
*
Cells (×10^6) per plate
1
2
3
4
5

Figure 4. Macrophage differentiation with M-CSF.
Purified monocytes and Bone marrow derived macrophages (BMDMs) on day 5 and day 12 of growth were stained with antibody against Ly6C, F4/80, Ly6G and CD11b as indicated and assessed through flow cytometry (A). The MFI expression of CD11b was calculated using Graphpad prism 7 (B). BMDMs on day 5 and day 12 of growth were scraped from petri dish and counted using haemocytometer (C).


A
Monocytes
**
%Cell viability
100
75
50
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0
Control
BP
BP+Nec-1
B
Day 5
Day 12
****
% Cell Viability
100
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Control
BP
BP+Nec-1
Control
BP
BP+Nec-1
C
Day 5
Day 12
% Cell Viability
100
75
50
25
0
6
24
Time (hrs) after BP
D
Control
BP
BP+Nec-1
Day 5
Day 12

Figure 5. Macrophages develop resistance to SMAC mimetic (Birinapant) induced cell death as they differentiate. Monocytes and BMDMs (at day 5 and day 12 of growth) from WT (C57BL/6) mice were treated with SMAC mimetic, Birinapant (BP) (10μM) with and without necrostatin-1 (Nec-1) (10μM). After 24 hours incubation, the cell viability was measured by MTT assay (A, B). Macrophages at day 5 and day 12 of differentiation treated with BP (10μM) were examined for the cell viability using Hoechst (Hoechst: blue for dead and live cells) and propidium iodide (PI: red for only dead cells) co-staining. Cells in pink color indicate dead cells and spots in blue indicate live cells (D). Graphs show the percentage of viable cells ±SEM relative to control (untreated cells). All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

4.1.3. BP induced cell death is dependent on TNF-R1 signaling

TNF-α and IFN-β, both are the critical cytokines in cell death pathways, homeostasis and inflammation (Ivashkiv and Donlin, 2014; Aggrawal, 2003). Their binding to their respective ligands TNF-R1, R2 and IFNAR1 induces a diverse array of immune responses. We therefore evaluated the contribution of TNF-R and IFNAR1 signaling in BP-induced cell death of macrophages. Bone marrow from WT mice, and mice lacking individual subunits of the TNF-R (TNF-R1-/- and TNF-R2-/-) were used to generate macrophages. D5 macrophages were treated with BP and the cell viability was measured at 24 hours by MTT assay. Interestingly, TNF-R1-/-, but not TNF-R2-/- macrophages were completely resistant to BP-induced cell death (Fig. 6A). Since the cell death was dependent on TNF-R1 receptor, we then tested the role of autocrine TNF-α production in BP-induced cell death. BMDMs of WT and TNF-α -/- mice were treated with BP and the cell viability was assessed. TNF-α-/- macrophages showed complete resistance to cell death induced by BP compared to its WT control (Fig. 6B). Then we evaluated the TNF-α expression in D5 and D12 macrophages by taking the supernatants from the respective wells and assessing it with a bioassay using WEHI cells, the murine fibrosarcoma cell line. Surprisingly, there was no significant difference in the TNF-α production between D5 and D12 macrophages (Fig. 6C).

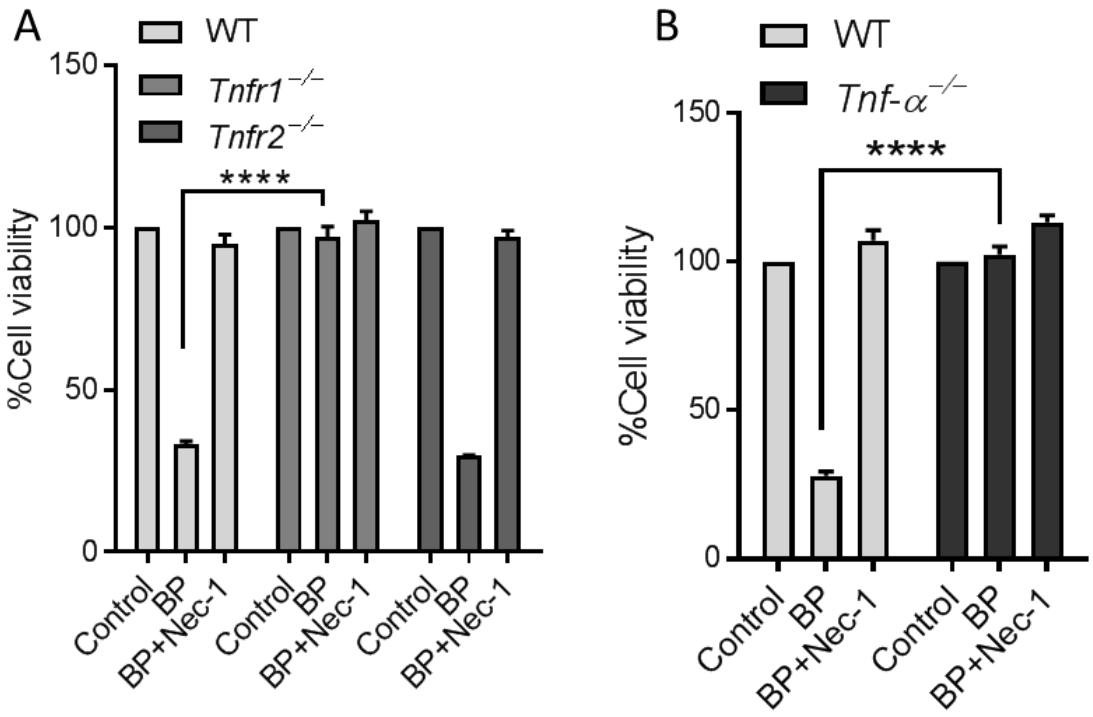
We also evaluated the impact of IFNAR1-signaling in BP-induced cell death using macrophages from IFNAR1-/- mice. Deficiency of IFNAR1 didn't rescue the cells from BP induced cell death (Appendix-Fig. IIA). Our data also showed that the adaptor protein TRIF has a minor role in ripoptosome-induced cell death, and MyD88 does not have any role (Appendix-Fig. IIB, C). These data suggest that TNF-α induced TNF-R1 signaling and subsequent downstream signaling is a crucial and indispensable mechanism for BP induced

cell death of macrophages, however there is no difference in the TNF-α expression in the macrophages at two stages of differentiation.

4.1.4. The role of RipK1 and RipK3 in ripoptosome signaling

The kinases, RipK1 and RipK3 have been reported to be the critical mediators of necroptosis (Pasparakis and Vandenabeele, 2015). SM has been known to induce apoptosis that is dependent on the kinase activity of RipK1 (Tenev et al., 2011). In conjunction with the caspase inhibitor, zVAD, SM also induces necroptosis that is dependent on the kinase activity of RipK1 and RipK3 (McComb et al., 2014). Thus we looked at role of these kinases in BP induced cell death of macrophages during their differentiation. Macrophages were derived from the bone marrow cells of WT, $RipK1^{K45A}$ (having dysfunctional kinase activity due to a K45A mutation) and RipK3 null mice and treated with BP+/- Nec-1 on day 5 of differentiation. Cell viability was measured at 24 hours by MTT assay. While RipK3 deficient macrophages showed significant resistance (Fig. 7A), RipK1 kinase inactive macrophages showed complete resistance to cell death compared to that of WT macrophages (Fig. 7B). Further, to investigate the role of kinase activity of RipK3 in BP induced cell death we treated monocytes and D5 and D12 macrophages with GSK 872, which inhibits the kinase activity of RipK3. Rather than rescuing BP induced cell death, treatment of cells with GSK 872 enhanced the cell death of macrophages following BP treatment (Fig. 7C, D, and E). Thus the kinase activity of RipK1 and RipK3 appears to have opposite impact on BP induced cell death of macrophages. Since RipK3 promotes BP induced cell death that is

Day 5 Macrophages



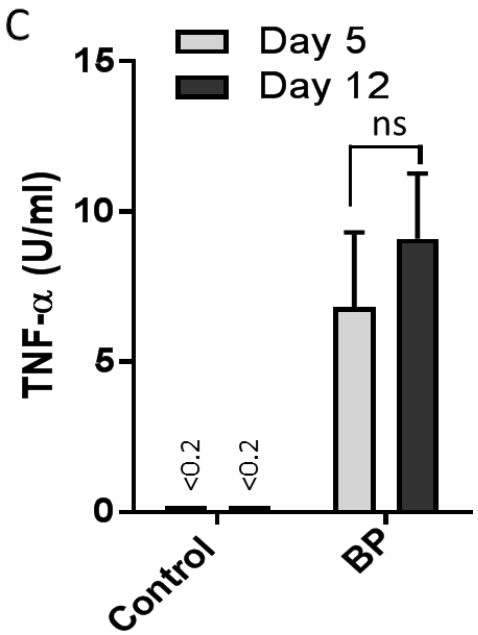


Figure 6. TNF-α/TNF-R1 signaling is indispensable for BP induced cell death in macrophages. D5 BMDMs from WT (C57BL/6), TNF-R1-/-, TNF-R2-/- and TNF-α-/- mice were treated with BP (10µM) with and without Nec-1 (10µM). After 24 hours incubation, the cell viability was measured by MTT assay (A, B). The supernatants of D5 and D12 macrophages treated with BP (10µM) for 24 hours were assessed for TNF-α production using the bioassay on WEHI cells (C). The assay detects the cytotoxic effect of the TNF-α, present in the supernatant, on WEHI cells and the cell viability is measured by MTT assay. The cell death is directly proportional to the TNF-α present in the supernatant. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

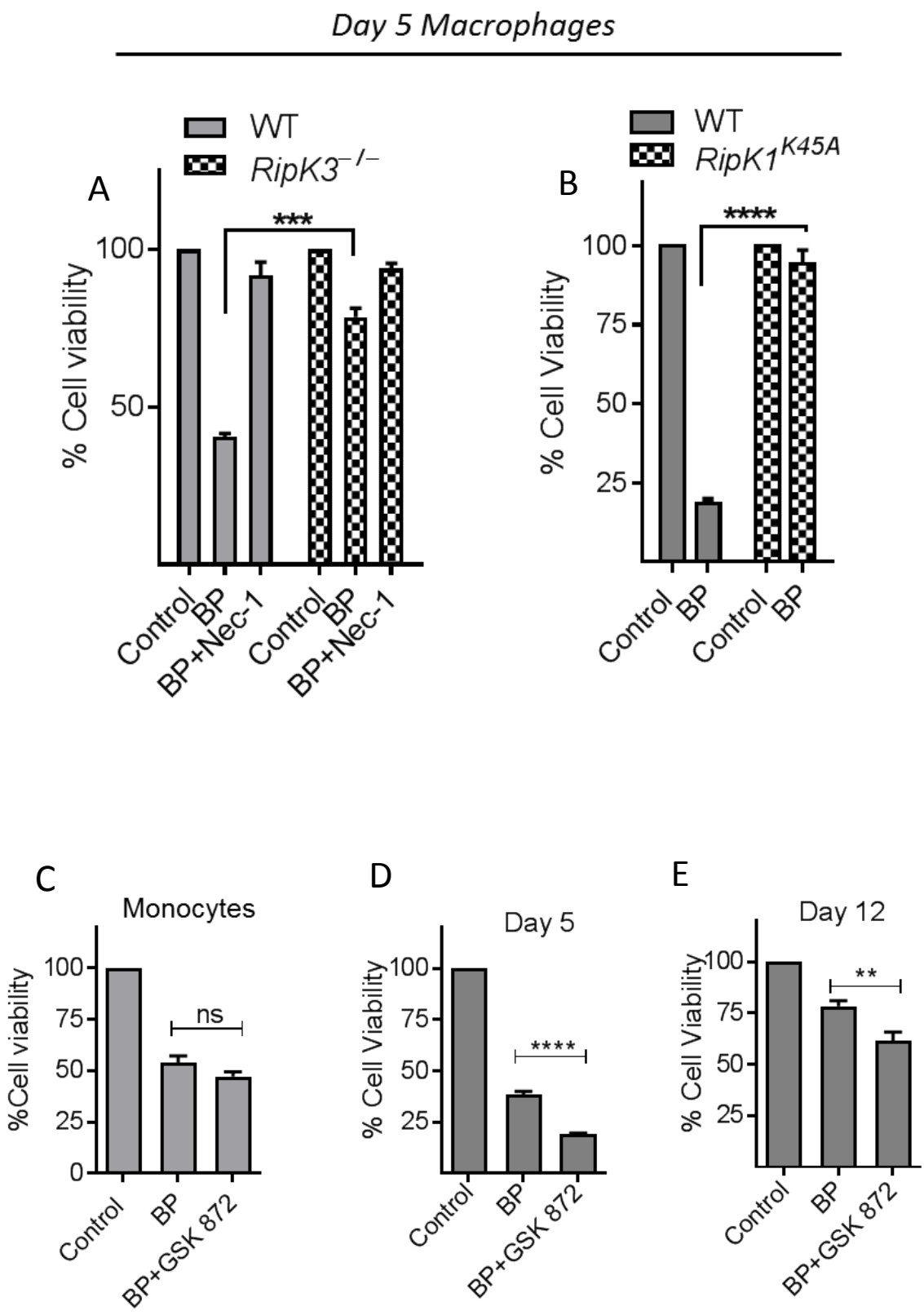

Day 5 Macrophages
A
WT
RipK3−/−
% Cell viability
100
50
***
Control
BP
BP+Nec-1
Control
BP
BP+Nec-1
B
WT
RipK1K45A
% Cell Viability
100
75
50
25
****
Control
BP
Control
BP
C
Monocytes
%Cell viability
100
75
50
25
0
ns
Control
BP
BP+GSK 872
D
Day 5
% Cell Viability
100
75
50
25
****
Control
BP
BP+GSK 872
E
Day 12
% Cell Viability
100
75
50
25
**
Control
BP
BP+GSK 872

Figure 7. The kinase region of RipK1 and the scaffold of RipK3 are required for BP induced cell death. D5 BMDMs from WT (C57BL/6), RipK3-/- and RipK1^{K45A} mice were treated with BP (10µM) with and without Nec-1 (10µM). After 24 hours incubation, the cell viability was measured by MTT assay (A, B). Monocytes, and D5 and D12 BMDMs were treated with BP (10µM) +/- GSK 872 and the cell viability measured by MTT assay (C, D and E). Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

independent of its kinase region, this suggests that RipK3 must mediate a scaffolding function during cell death induced by the SMAC mimetic.

4.1.5. Phosphorylation of RipK1 is impaired by prolonged differentiation of macrophages

To delve more into the mechanism of resistance of macrophages to cell death following prolonged differentiation, we investigated the activation (or lack thereof) of key proteins, kinases and transcription factors that are known to be involved in ripoptosome-induced cell death. We collected cell lysates from D5 and D12 WT BMDMs that were treated with BP for varying time intervals, and performed western blot analysis. We first looked at the activation of RipK1 and RipK3. The antibodies that we used for the measurement of the activation of RipK1 and RipK3 can detect the phosphorylated and unphosphorylated forms of these proteins. The slower migrating band represents the phosphorylated bands of RipK1 and RipK3 (McComb et al., 2014). Following BP treatment, phosphorylation of RipK1 was detected at 3 hours post treatment in D5 macrophages. This phosphorylation of RipK1 was completely abolished in D12 macrophages (Fig. 8A). We further evaluated activation of proteins by performing densitometric analysis of western blots. The densitometry analysis clearly revealed the impairment in RipK1 phosphorylation in D12 macrophages (Fig. 8C). In contrast to RipK1, the phosphorylation of RipK3 was not observed in D5 and D12 macrophages treated with BP (Fig. 8A, D). There was no change in the total protein levels of RipK1 and RipK3 (Fig. 8B, D). The expression level of FADD, which is an important protein of ripoptosome complex, was not influenced by BP treatment of D5 and D12 macrophages (Fig. 8A). Thus, the prolonged differentiation of macrophages seems to impact on the phosphorylation of RipK1 but not RipK3.

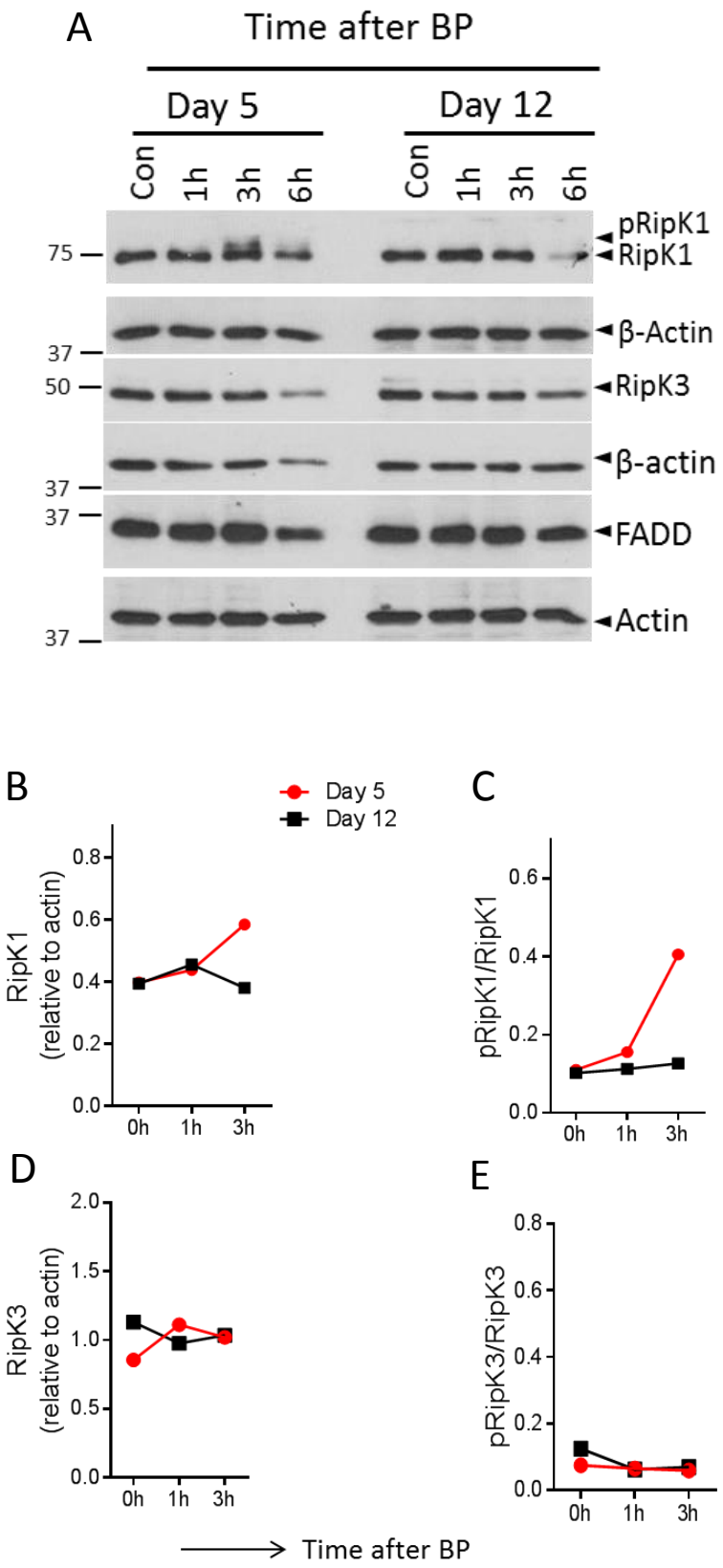

A
Time after BP
Day 5
Day 12
Con
1h
3h
6h
Con
1h
3h
6h
75
37
50
37
37
37
pRipK1
RipK1
β-Actin
RipK3
β-actin
FADD
Actin
B
Day 5
Day 12
RipK1 (relative to actin)
0.0
0.2
0.4
0.6
0.8
0h
1h
3h
C
pRipK1/RipK1
0.0
0.2
0.4
0.6
0h
1h
3h
D
RipK3 (relative to actin)
0.0
0.5
1.0
1.5
2.0
0h
1h
3h
E
pRipK3/RipK3
0.0
0.2
0.4
0.6
0.8
0h
1h
3h
Time after BP

Figure 8. RipK3 and FADD are not impacted while RipK1 phosphorylation is abolished with prolonged differentiation of macrophages. Lysates were collected from BP (10μM) treated WT macrophages over the course of 6 hours (as indicated), on day 5 and day 12 of differentiation to measure RipK1, RipK3 and FADD by western blot (A). The buffer used for lysing was SDS buffer. Densitometric ratios of total RipK1 (B), phosphorylated RipK1 to unphosphorylated RipK1 (pRipK1/RipK1) (C), total RipK3 (D) and phosphorylated RipK3 to unphosphorylated RipK3 (pRipK3/RipK3) (E) were calculated. The densitometric analysis is shown only for the first three-hour post treatment for each protein. All western blot experiments were done at least three times in three different mice.

4.1.6. Prolonged differentiation of macrophages upregulates anti-apoptotic proteins

To evaluate the mechanism of resistance of macrophages to cell death following ripoptosome signaling, we determined the expression of anti-apoptotic or pro-survival proteins following prolonged differentiation of macrophages. In many human cancers, the alterations in the IAP proteins such as cIAP1, cIAP2 and XIAP has been associated with chemoresistance (Hunter et al., 2007). Similarly, $cFLIP_L$ (the long isoform of cFLIP) has been reported to interact with caspase 8 and form a caspase 8/$FLIP_L$ heterodimer which restricts the activity of caspase 8 (Wilson et al., 2009). We collected the lysates from D5 and D12 WT BMDMs, treated with BP and performed western blot analysis of various proteins. The antibody that we used for detection of cIAPs detects both cIAP1 and cIAP2, and fails to discriminate between cIAP1 and cIAP2 as the two proteins have similar molecular weights (cIAP1-62 kDa, cIAP2-70 kDa). As expected, treatment of macrophages with BP resulted in complete degradation of cIAPs within one hour of treatment of D5 macrophages (Fig. 9A). Similar results were obtained with D12 macrophages (Fig. 9A). Interestingly, D12 macrophages expressed higher levels of XIAP and $cFLIP_L$ following BP treatment in comparison to D5 macrophages (Fig. 9A). Densitometric analysis was performed for cIAPs, XIAP and $cFLIP_L$ levels, which were normalized to actin (Fig. 9B, C and D). These data indicated that the increased levels of the anti-apoptotic proteins XIAP and $cFLIP_L$ correlated with the resistance against ripoptosome signaling during macrophage differentiation.

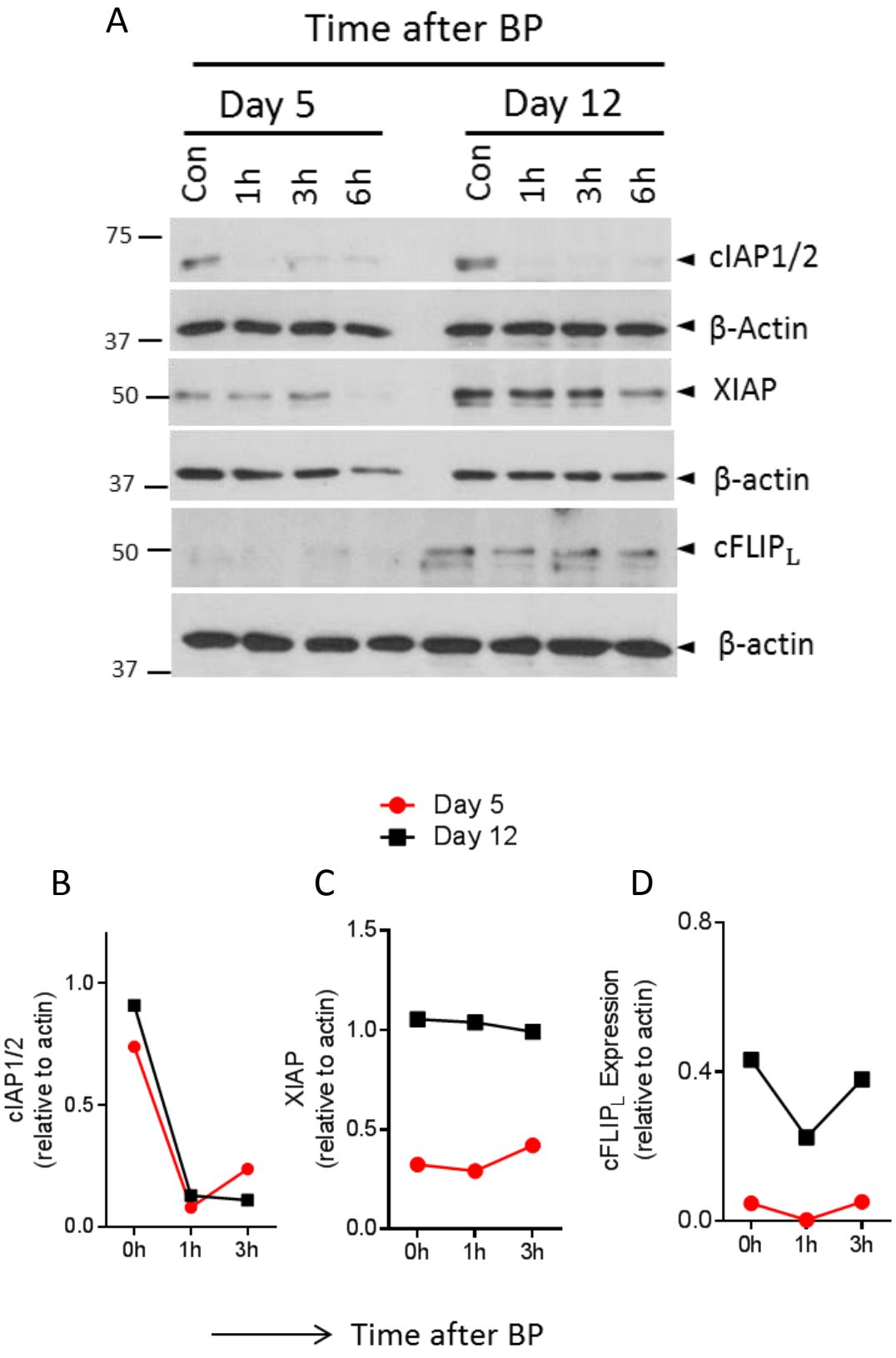

A
Time after BP
Day 5
Day 12
Con
1h
3h
6h
Con
1h
3h
6h
75
37
50
37
50
37
cIAP1/2
β-Actin
XIAP
β-actin
cFLIP$_L$
β-actin
Day 5
Day 12
B
cIAP1/2 (relative to actin)
1.0
0.5
0.0
0h
1h
3h
C
XIAP (relative to actin)
1.5
1.0
0.5
0.0
0h
1h
3h
D
cFLIP$_L$ Expression (relative to actin)
0.8
0.4
0.0
0h
1h
3h
Time after BP

Figure 9. XIAP and cFLIP$_L$ are upregulated with prolonged differentiation of macrophages. Lysates were collected from BP (10μM) treated WT macrophages over the course of 6 hours (as indicated), on day 5 and day 12 of differentiation to measure cIAPs, XIAP, cFLIP$_L$ and β-actin by western blot (A). The buffer used for lysing was SDS buffer. Densitometric ratios of cIAPs (B), XIAP (C) and cFLIP$_L$ (D) were calculated by normalising to actin. The densitometric analysis is shown only for the first three-hour post treatment for each protein. All western blot experiments were done at least three times in three different mice.

4.1.7. Impact of prolonged differentiation of macrophages on NF-kB signaling

The upregulation of the anti-apoptotic proteins XIAP and $cFLIP_L$ that we observed with macrophage differentiation may be related to the impact on the "master transcription factor, NF-kB" and the MAPKs. Thus we investigated the impact of macrophage differentiation on the activation of NF-kB and p38 MAPK. NF-kB is a member of transcription factors that are important in pro-survival and inflammatory response and cIAPs and $cFLIP_L$ are the transcriptional targets of canonical NF-kB pathway (Kreuz et al., 2001a). The alternative, non-canonical NF-kB pathway is known to induce TNF-α expression in response to cIAPs degradation (Chan et al., 2015). We thus evaluated the activation of canonical and non-canonical NF-kB pathway following BP treatment of cells using antibodies that specifically bind the activated (phosphorylated) versions of p65 (canonical) and p100 (non-canonical). In response to BP treatment, expression of the phosphorylated p65 was increased in D5 macrophages and this activation was reduced in D12 macrophages (Fig. 10A). In contrast, the expression of the phosphorylated p100 was higher in D12 macrophages in comparison to D5 macrophages (Fig. 10B). Densitometric analysis was performed for the expression of phosphorylated p65 in comparison to total p65 (pp65/p65) (Fig. 10C) and the expression of phosphorylated p100 in comparison to total p100 (pp100/p100) (Fig. 10F). Results clearly indicate that prolonged differentiation of macrophages results in a reduction in the canonical NF-kB activation but enhancement of the non-canonical NF-kB signaling following treatment with BP.

In addition to NF-kB, TNF-R1 signaling also activates the MAP kinases (MAPKs). p38 MAPK and its downstream kinase MK2 have been implicated in many signaling cascades in response to cytokines and other stress stimuli (Zarubin and Han, 2005). To investigate the role of these kinases in BP induced cell death, we collected the lysates after

treatment of cells with BP over the course of 6 hours and probed the membranes with antibody against phosphorylated and total p38 MAPK and MK2. Potent activation (phosphorylation) of the p38 MAPK and MK2 was seen in D5 macrophages, but this activation was highly attenuated in D12 macrophages (Fig. 10A). Densitometric analysis of phosphorylated versus total p38 MAPK and MK2 clearly showed the higher activation of p38 MAPK and MK2 in D5 macrophages (Fig 10D, E).

These results indicate that BP treatment of D5 macrophages results in potent activation of the canonical NF-kB and p38 MAPK signaling, whereas prolonged differentiation of macrophages shifts the signaling more towards higher activation of the non-canonical NF-kB pathway and a reduction in the p38 MAPK signaling pathway.

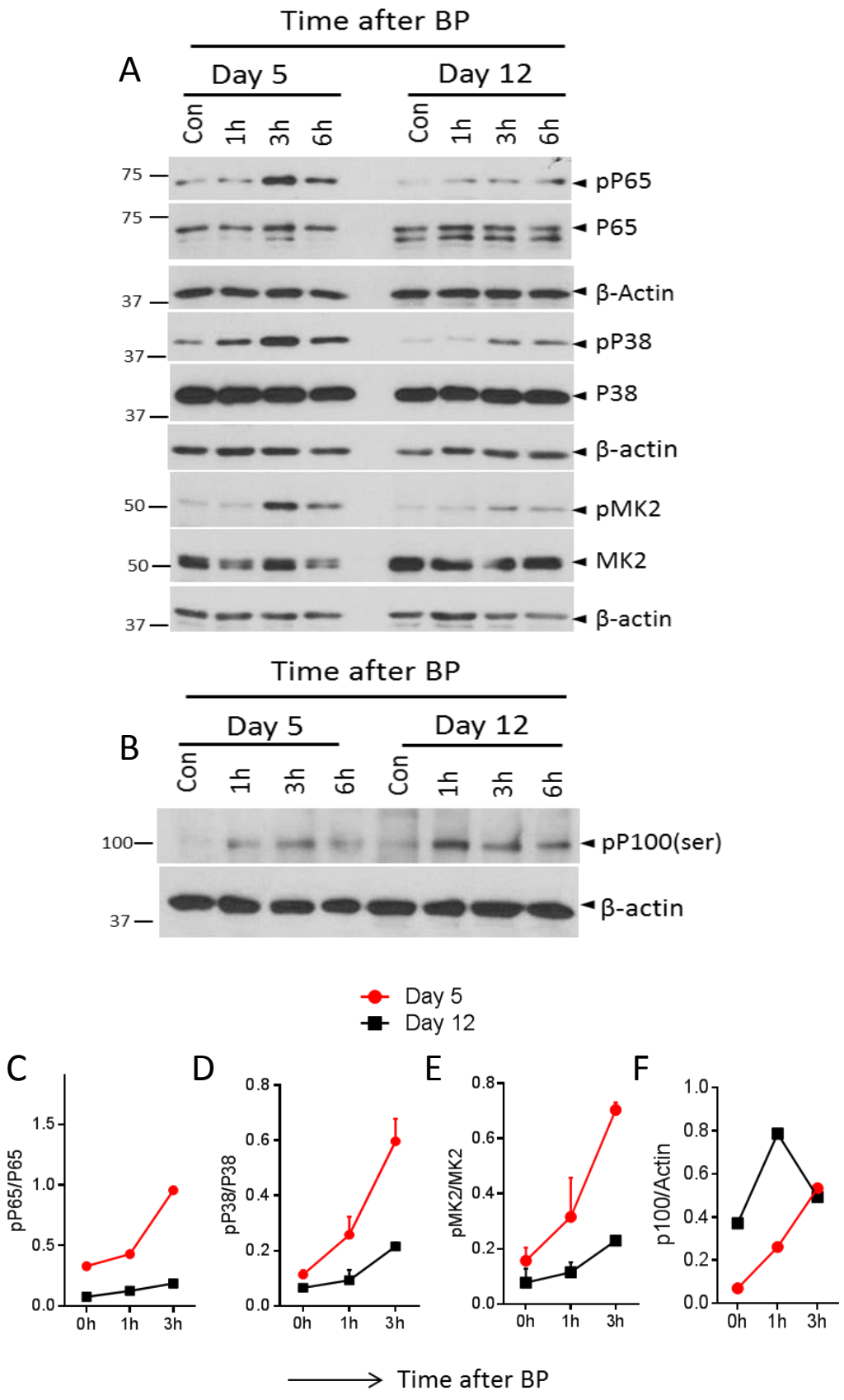
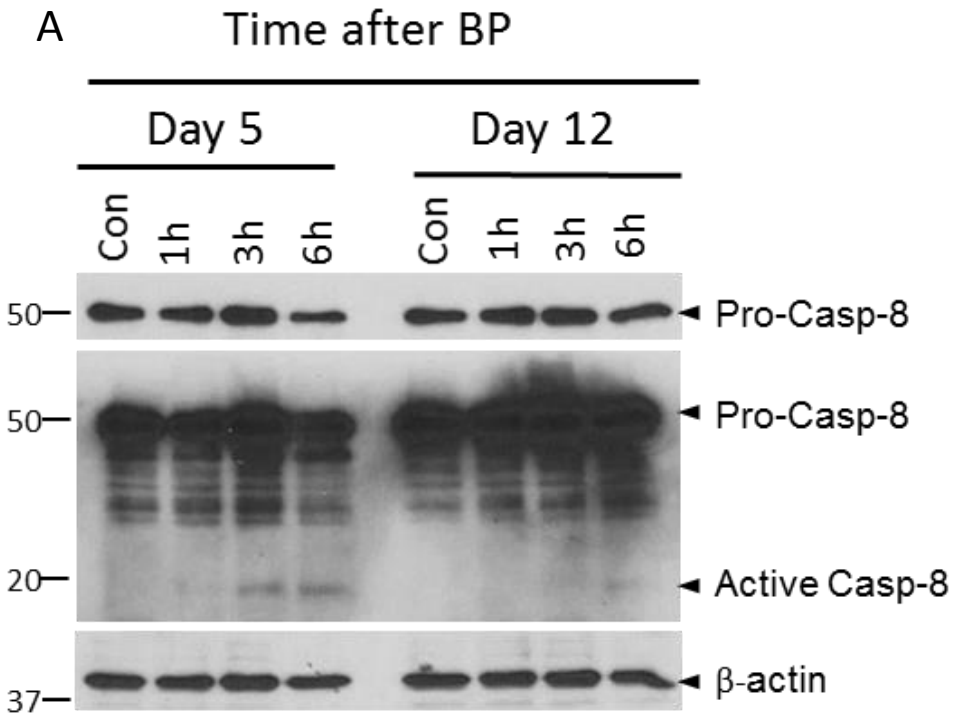

A
Time after BP
Day 5
Day 12
Con
1h
3h
6h
75
pP65
P65
β-Actin
37
pP38
P38
β-actin
50
pMK2
MK2
B
pP100(ser)
C
pP65/P65
D
pP38/P38
E
pMK2/MK2
F
p100/Actin
0h
Time after BP

Figure 10. Activation of canonical NF-kB, p38 MAPK and MK2 is impaired in D12 macrophages. Lysates were collected from BP (10μM) treated WT macrophages over the course of 6 hours (as indicated), on day 5 and day 12 of differentiation to measure pp65, p65, pP38 MAPK, p38, pMK2, MK2, p100 and β-actin by western blot (A, B). The buffer used for lysing was SDS buffer. Densitometric ratios of pp65/p65 (C), pP38 MAPK/total p38 MAPK (D), pMK2/MK2 (E) and pp100/actin (B) were calculated. The densitometric analysis is shown only for the first three-hour post treatment for each protein. All western blot experiments were done at least three times in three different mice.

4.1.8. Prolonged differentiation of macrophages compromises activation of caspase 8

My results demonstrated that prolonged differentiation of macrophages resulted in resistance of macrophages to cell death in response to ripoptosome activation. Since ripoptosome-induced cell death is mediated by caspase 8 activation (Tenev et al., 2011), we evaluated the activation of caspase 8 in macrophages that were differentiated till day 12. We collected lysates from WT BMDMs treated with BP, on day 5 and day 12 of differentiation and performed western blot analysis of caspase 8. The antibody against caspase 8 detects both the procaspase 8 (molecular weight 57kDa) and the processed caspase 8 or p18 (molecular weight 18kDa). Interestingly, the cleaved form (active) of caspase 8 (p18) was detectable in D5 macrophages and this was substantially reduced in D12 macrophages (Fig. 11A). In addition to measuring the activation of caspase 8 by western blotting, we also measured the activity of caspase 8 using a Caspase-Glo® 8 luminescent assay. The luminescent assay detects luminescence, which is caused by cleavage of the synthetic substrate by caspase 8. Thus the luminescence generated is directly proportional to the activity of caspase 8. D5 cells treated with BP showed high caspase 8 activity in comparison to D12 cells (Fig. 11B). When the cells were treated with BP along with the caspase 8 inhibitor Z-IETD, the caspase 8 activity was reduced to basal levels similar to untreated cells, indicating the specificity of caspase 8 assay (Fig. 11B). These data indicate that the processing and activation of caspase 8 is impaired with the prolonged differentiation of macrophages and this impairment in caspase 8 activity can be correlated to the resistance to macrophages to cell death in response to ripoptosome signaling.


A
Time after BP
Day 5
Day 12
Con
1h
3h
6h
Con
1h
3h
6h
50
Pro-Casp-8
50
Pro-Casp-8
20
Active Casp-8
37
β-actin

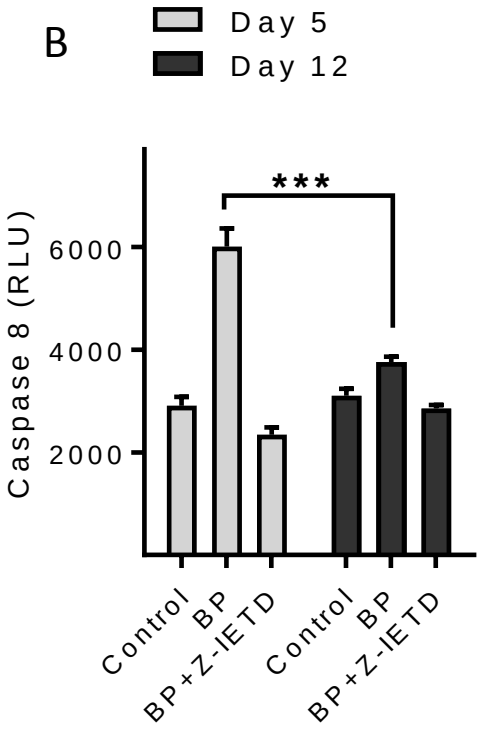

B
Day 5
Day 12
***
Caspase 8 (RLU)
6000
4000
2000
Control
BP
BP+Z-IETD
Control
BP
BP+Z-IETD

Figure 11. Day 12 macrophages have impaired caspase 8 activity. Lysates were collected from BP (10μM) treated WT macrophages over the course of 6 hours (as indicated), on day 5 and day 12 of differentiation to measure caspase 8 and β-actin by western blot (A). The buffer used for lysing was SDS buffer. Macrophages treated with BP (10μM) with and without Z-IETD (50μM) were subjected to Caspase-Glo® 8 assays and the luminescence were measured by the plate reader (B). All western blot and luminescent experiments were done at least three times in three different mice.

4.1.9. Prolonged differentiation of macrophages results in increased cFLIP$_L$-casp-8 interaction

The ripoptosome complex consists of RipK1, FADD, caspase 8 as the core components along with RipK3 and the isoforms of cFLIP (Tenev et al., 2011; Feoktistova et al., 2011). Having seen the impairment of caspase 8 processing and activity in D12 macrophages, we evaluated whether the differentiation of macrophages influences the interaction of caspase 8 with other proteins in the ripoptosome complex after treatment of the cells with BP. Macrophages were treated with BP for four hours, and caspase 8 was immunoprecipitated with an anti-caspase 8 antibody coupled to magnetic beads. Immunoprecipitates were then subjected to western blotting against RipK1, FADD, cFLIP$_L$, RipK1, caspase 8 and β-actin. As shown in Fig. 12 A, treatment of D5 macrophages with BP resulted in increased interaction of caspase 8 with RipK3 and FADD. With prolonged differentiation, there was reduction in the interaction of caspase 8 with RipK3 (Fig. 12A). Interaction of caspase 8 with FADD was not impacted by prolonged differentiation of macrophages. Interaction of RipK1 with caspase 8 was undetectable. Interestingly, we observed caspase 8-cFLIP$_L$ interaction only in D12 macrophages (Fig. 12A). Since we performed immunoprecipitation using an anti-caspase 8 antibody, caspase 8 was detected in all the samples. Actin was not detectable in the immunoprecipitates, which provided a positive validation of our immunoprecipitation protocol. We also measured the expression of ripoptosome signaling proteins in the cell extracts, without immunoprecipitations (input samples) (Fig. 12 B). These results indicate that the diminished caspase 8 and RipK3 interaction and the enhanced caspase 8 and cFLIP$_L$ interaction correlated with the resistance against ripoptosome signaling during macrophage differentiation.

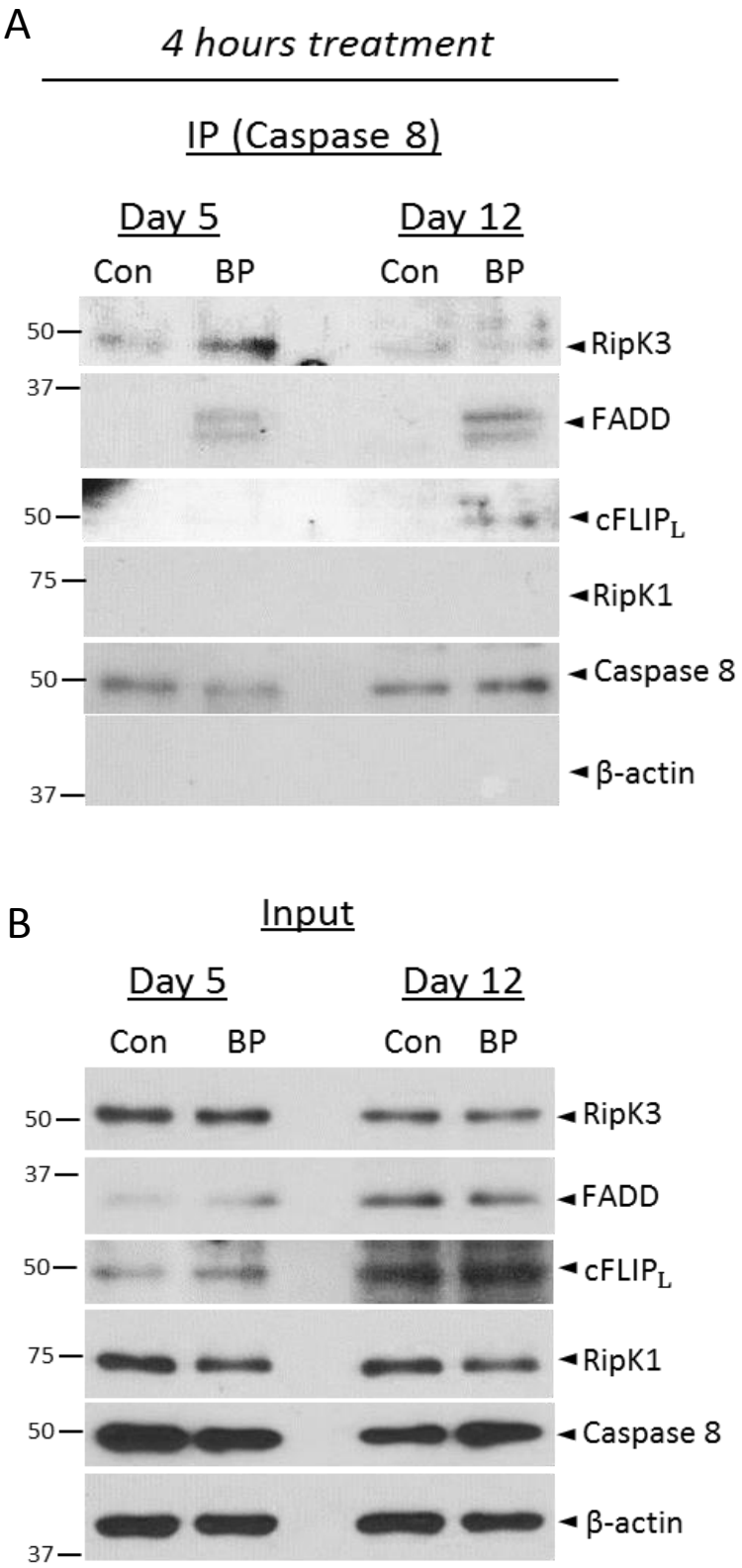

A
4 hours treatment
IP (Caspase 8)
Day 5
Day 12
Con
BP
Con
BP
50
RipK3
37
FADD
50
cFLIP$_L$
75
RipK1
50
Caspase 8
37
β-actin
B
Input
Day 5
Day 12
Con
BP
Con
BP
50
RipK3
37
FADD
50
cFLIP$_L$
75
RipK1
50
Caspase 8
37
β-actin

Figure 12. Caspase 8 interaction is enhanced with $cFLIP_L$ in day 12 macrophages. D5 and D12 macrophages were treated with BP for four hours. Cells were then lysed and immunoprecipitated using magnetic beads coupled with anti-caspase 8. The immunoprecipitates (A) and the total lysates from input control (B) were subjected to western blotting for the detection of RipK3, FADD, $cFLIP_L$, RipK1, caspase 8 and β-actin.

4.1.10. The kinase function of RipK1 is required for caspase activation and ripoptosome signaling

Since Nec-1 was able to inhibit BP induced cell death, and Nec-1 inhibits the kinase function of RipK1, we further evaluated the impact of the kinase function of RipK1 in BP induced cell death of macrophages. Caspase 8 and caspase 9 are the initiator caspases, which when activated cleave and activate the downstream caspases, caspase 3 and caspase 7. Activation of caspase 3/7 results in apoptotic cell death (Shiozaki et al., 2003). We evaluated the activation of these caspases during ripoptosome signaling and the impact of Rip1K kinase activity. We generated macrophages from WT and the RipK1^{K45A} (the kinase inactive) mice at day 5 of differentiation, treated them with BP for 3-6 hours and measured the activity of caspase 8, caspase 9 and caspase 3/7 using Caspase-Glo® 8 assay, Caspase-Glo® 9 assay and Caspase-Glo® 3/7 assay respectively. These bioassays detect luminescence, which is caused by caspase-mediated cleavage of the specific substrates. The luminescence generated is directly proportional to the caspase activity. In response to BP treatment, caspase 8 activity was induced in WT macrophages as expected and this activation was completely abrogated in RipK1^{K45A} macrophages (Fig. 13A). Interestingly, there was activation of caspase 9 and caspase 3/7 in WT macrophages treated with BP and the activation of these caspases was also abrogated in RipK1^{K45A} macrophages (Fig. 13B, C). Treatment of cells with the pan-caspase inhibitor zVAD (Fig. 13A), caspase 9 inhibitor Z-LEHD (Fig. 13B) and caspase 3/7 inhibitor Z-DEVD (Fig. 13C) reduced the caspase activity to basal levels, indicating the specificity of caspase luminescence assays. These data indicate that the kinase activity of RipK1 is indispensable for activation of caspase 8, caspase 9 and caspase 3/7 in macrophages during ripoptosome signaling.

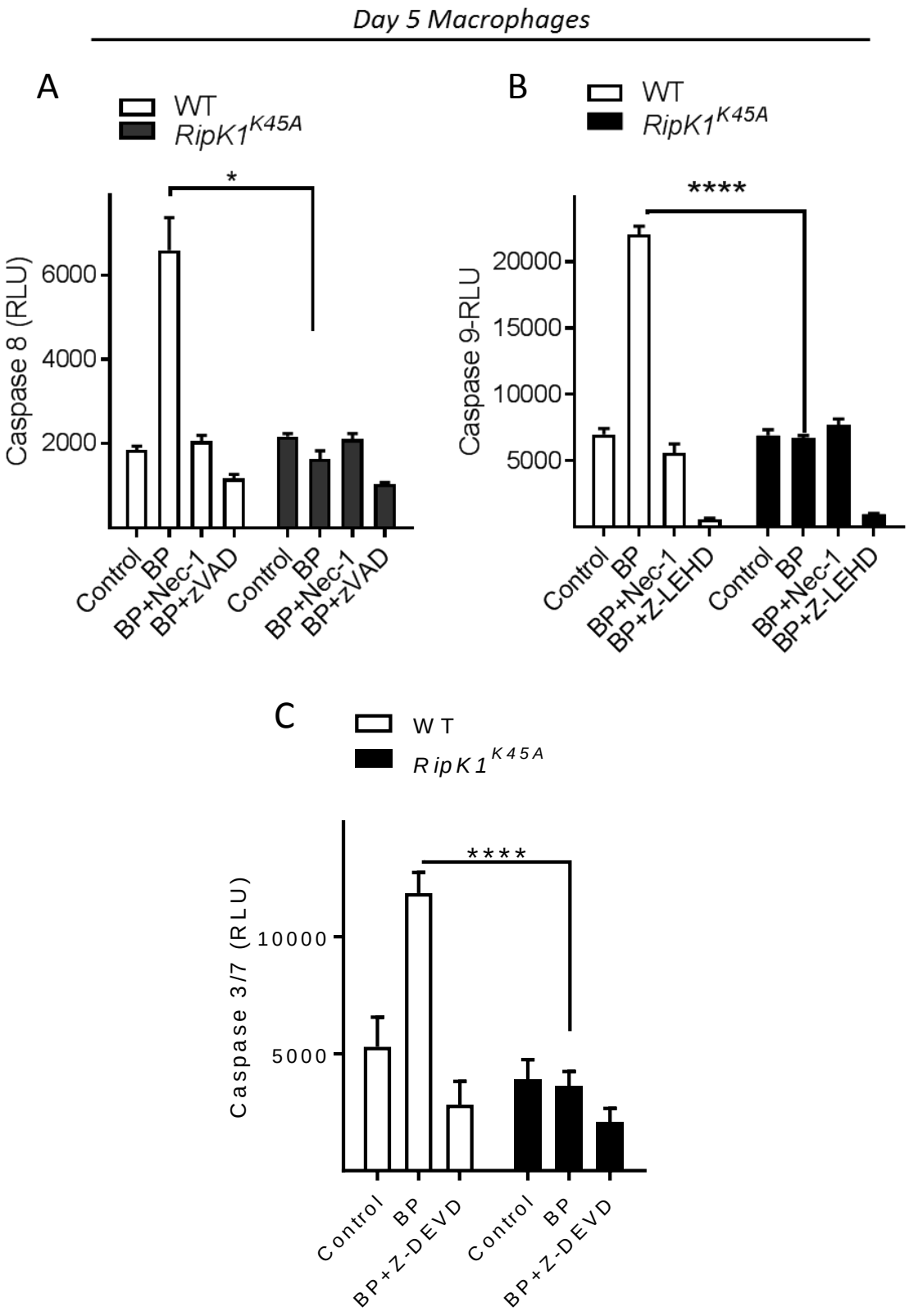

Day 5 Macrophages
A
WT
RipK1K45A
*
Caspase 8 (RLU)
6000
4000
2000
Control
BP
BP+Nec-1
BP+zVAD
Control
BP
BP+Nec-1
BP+zVAD
B
WT
RipK1K45A
****
Caspase 9-RLU
20000
15000
10000
5000
Control
BP
BP+Nec-1
BP+Z-LEHD
Control
BP
BP+Nec-1
BP+Z-LEHD
C
WT
RipK1K45A
****
Caspase 3/7 (RLU)
10000
5000
Control
BP
BP+Z-DEVD
Control
BP
BP+Z-DEVD

Figure 13. Caspase activities are impaired in RipK1 kinase deficiency. WT and RipK1^{K45A} macrophages were generated on day 5 of differentiation and treated with BP for 3-6 hours. Macrophages treated with BP (10μM) with and without zVAD (50μM) (A), Z-LEHD (50μM) (B) and Z-DEVD (50μM) (C) were subjected to Caspase-Glo® 8, Caspase-Glo® 9, Caspase-Glo® 3/7 assays and the luminescence was measured. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

4.1.11. Deficiency of XIAP restores the susceptibility of D12 macrophages to cell death by BP

XIAP is a member of IAP family of proteins, which directly inhibits the activity of caspase 9 and caspase 3/7 and thus negatively regulates the apoptotic cell death (Galban and Duckett, 2010). SMAC protein or SMAC mimetic inhibits XIAP, which results in restoration of apoptosis. XIAP is also known to regulate ripoptosome signaling (Tenev et al., 2011). In our previous results, we observed that prolonged differentiation of macrophages results in elevation of XIAP levels. We were therefore interested in determining whether this elevation of XIAP levels in highly differentiated macrophages was responsible for their resistance to BP induced cell death. BMDMs were generated from WT and XIAP-/- mice and D5 and D12 macrophages were treated with BP. The cell viability was assessed by MTT assay. There was enhancement of cell death in XIAP-/- D5 macrophages compared to D5 WT macrophages (Fig. 14A). Interestingly, the resistance of D12 macrophages to cell death in response to BP treatment was abrogated in XIAP-/- macrophages (Fig. 14A). We also measured the activity of caspase 8, caspase 9 and caspase 3/7 by luminescence bioassays in WT and XIAP-/- macrophages. While the activity of caspase-8 was reduced in D12 WT macrophages, the activity of caspase 8 was restored in D12 XIAP-/- macrophages (Fig. 14B). Similarly, caspase 9 and caspase 3/7 activity was significantly enhanced in XIAP-/- macrophages compared to WT macrophages (Fig. 14C, D). Treatment of cells with caspase 8 inhibitor Z-IETD (Fig. 14A), pan-caspase inhibitor (Fig. 14B, C) reduced the caspase activity to basal levels. These data indicate that the increase in XIAP levels observed in macrophages following prolonged differentiation results in their resistance to ripoptosome-induced cell death.

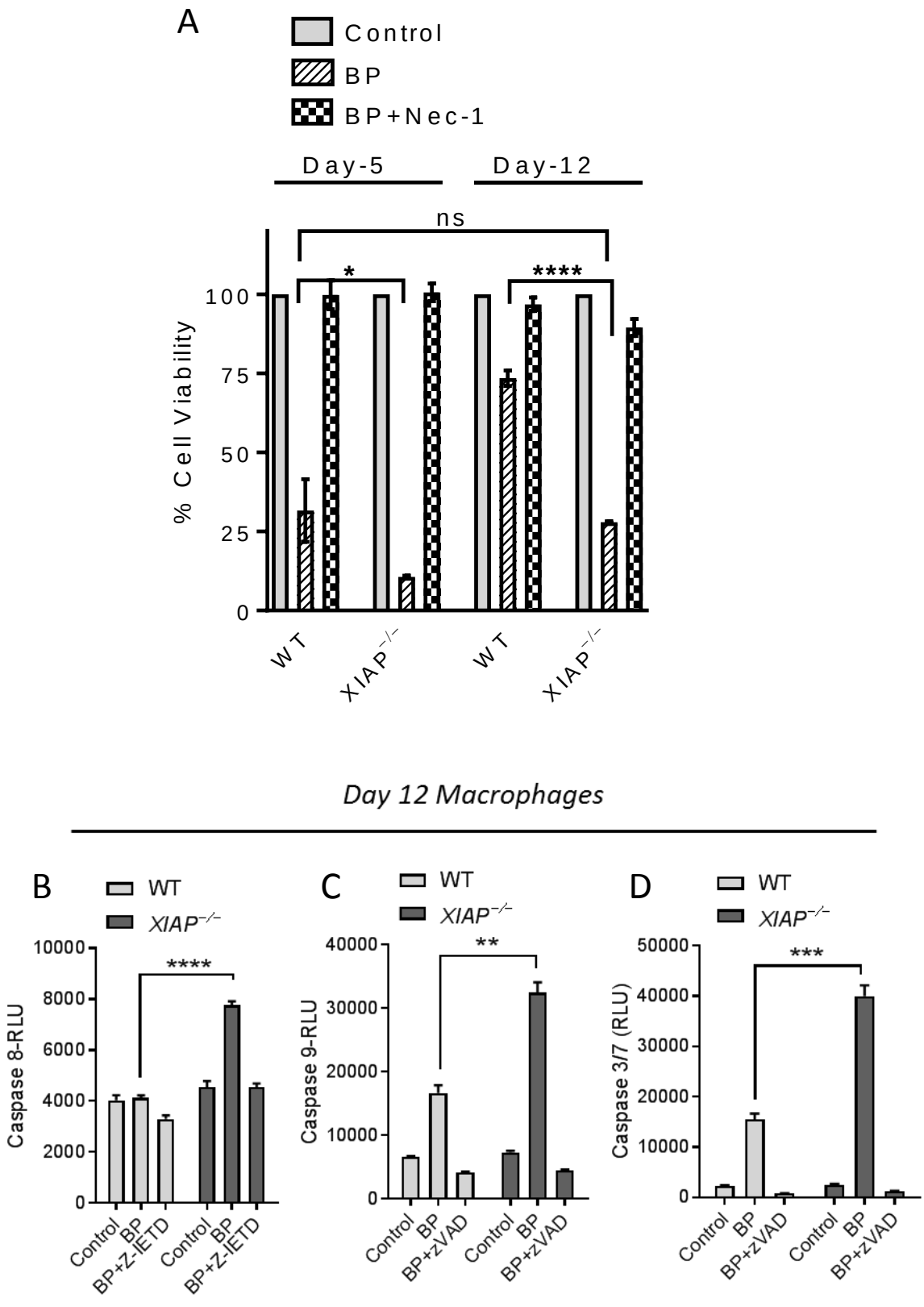

A
Control
BP
BP+Nec-1
Day-5
Day-12
ns
*
****
% Cell Viability
100
75
50
25
0
WT
XIAP−/−
WT
XIAP−/−
Day 12 Macrophages
B
WT
XIAP−/−
****
Caspase 8-RLU
10000
8000
6000
4000
2000
0
Control
BP
BP+Z-IETD
Control
BP
BP+Z-IETD
C
WT
XIAP−/−
**
Caspase 9-RLU
40000
30000
20000
10000
0
Control
BP
BP+zVAD
Control
BP
BP+zVAD
D
WT
XIAP−/−
***
Caspase 3/7 (RLU)
50000
40000
30000
20000
10000
0
Control
BP
BP+zVAD
Control
BP
BP+zVAD

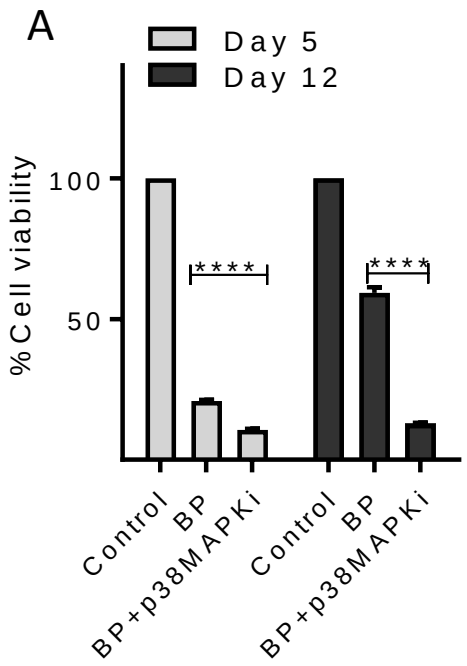
Figure 14. Day 12 macrophages are susceptible to cell death in XIAP deficiency. BMDMs from WT (C57BL/6) and XIAP -/- mice were treated with BP (10µM) with and without Nec-1 (10µM). After 24 hours incubation, the cell viability was measured by MTT assay (A). Macrophages treated with BP (10µM) with and without Z-IETD (50µM) (B) and zVAD (50µM) (C and D) were assessed by Caspase-Glo® 8, Caspase-Glo® 9, Caspase-Glo® 3/7 assays and the luminescence was measured. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

4.1.12. Inhibition of p38 MAPK restores BP induced cell death of D12 macrophages

It has been previously reported that p38 MAPK can promote cell survival and cell death that is dependent on the cell type involved (Zarubin and Han, 2005). We investigated the role of p38 MAPK in the context of ripoptosome signaling in macrophages. D5 and D12 WT BMDMs were treated with BP, with and without p38 MAPK inhibitor, LY2228820. It has been previously reported that p38 has four isoforms (α, β, γ and δ) and that LY2228820 inhibits only the p38α and p38β isoforms. We used this inhibitor since these two isoforms are the predominant ones and ubiquitously expressed in various cells. The cell death induced by BP in D5 macrophages was enhanced when p38 MAPK was inhibited (Fig. 15A). Interestingly, the resistance to cell death developed by D12 macrophages was abrogated by treatment with the p38 MAPK inhibitor (Fig. 15A). The enhanced cell death induced by p38 MAPK inhibitor was rescued by the RipK1 kinase inhibitor Nec-1, but was not by the RipK3 kinase inhibitor GSK 872 (Fig. 15B), suggesting that p38 MAPK inhibitor enhances ripoptosome-induced cell death. To further confirm the cell death to be RipK1 kinase dependent, we used D5 BMDMs of WT and RipK1^{K45A} mice and treated them with BP with and without p38 MAPK inhibitor. Treatment of RipK1^{K45A} macrophages with p38 MAPK inhibitor failed to induce cell death following BP treatment (Fig. 15C). Taken together, these results indicate that p38 MAPK signaling inhibits ripoptosome-induced cell death of macrophages.

4.1.13. P38 MAPK restricts caspase 8 activation in differentiated macrophages

Since, inhibition of p38 MAPK signaling abrogated the resistance of macrophages to BP induced cell death, we investigated the mechanism involved. We first evaluated the


A
Day 5
Day 12
% Cell viability
100
50
****
****
Control
BP
BP+p38MAPKi
Control
BP
BP+p38MAPKi

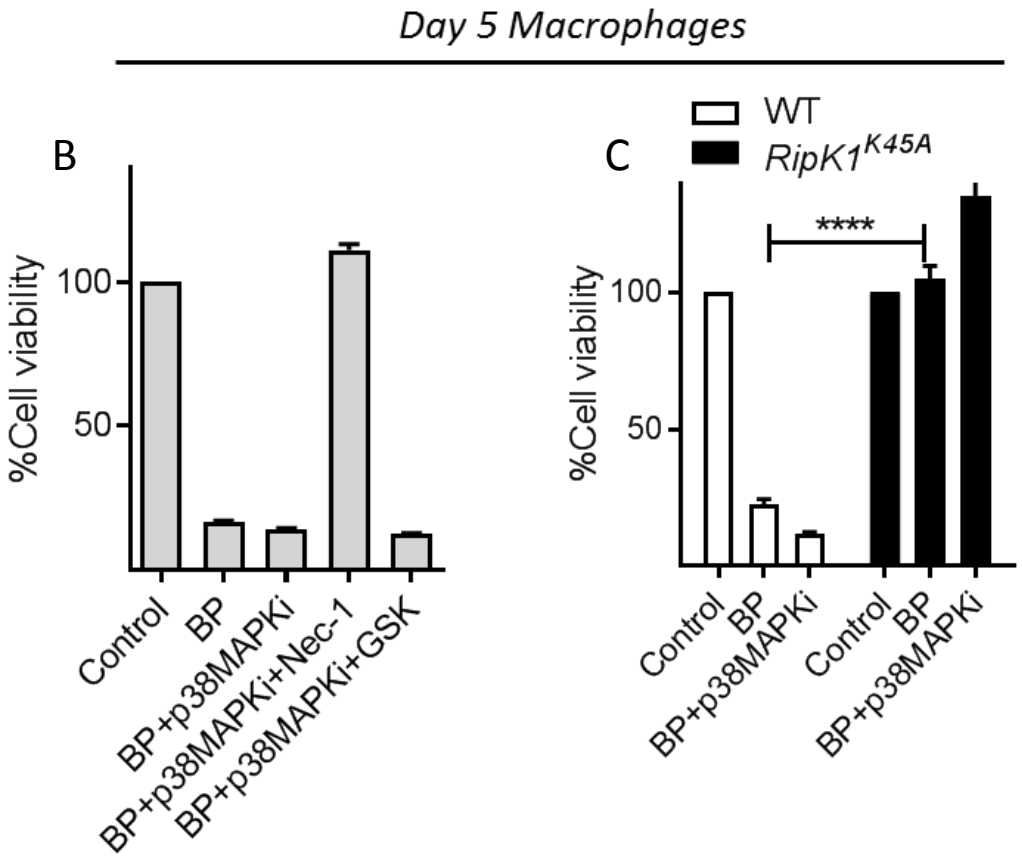

Day 5 Macrophages
B
%Cell viability
100
50
Control
BP
BP+p38MAPKi
BP+p38MAPKi+Nec-1
BP+p38MAPKi+GSK
C
WT
RipK1K45A
****
%Cell viability
100
50
Control
BP
BP+p38MAPKi
Control
BP
BP+p38MAPKi

Figure 15. P38 MAPK inhibitor enhances the cell death induced by BP in D5 and D12 macrophages. WT macrophages were generated on D5 and D12 of differentiation and treated with BP (10μM) +/- p38 MAPK inhibitor (LY2228820) (5μM) and cell viability was measured via MTT assay at 24 hours (A). D5 WT macrophages were treated with BP (10μM), p38 MAPK inhibitor (5μM), Nec-1 (10μM) and GSK 872 (5μM) as indicated and cell viability measured by MTT assay (B). BMDMs generated from WT and RipK1^{K45A} mice at day 5 of differentiation was treated with BP (10μM) +/-p38 MAPK inhibitor and cell viability measured by MTT assay (C). Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

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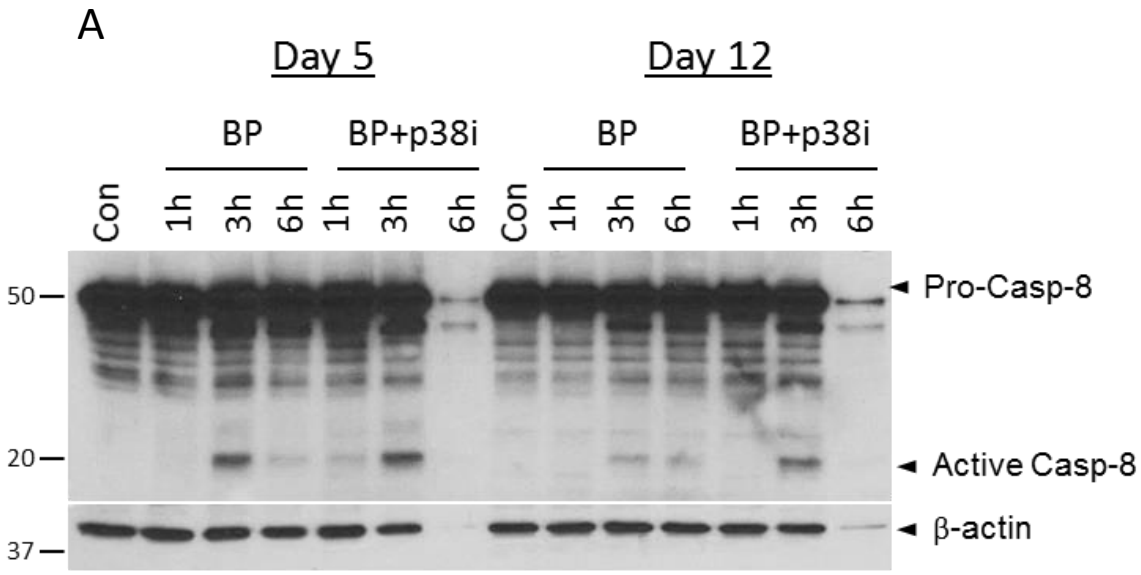
impact of p38 MAPK inhibitor on caspase 8 activation following treatment of cells with BP. It has been previously reported that p38 MAPK signaling inactivates caspase 8 by phosphorylation at Ser 364 in neutrophils (Alvarado-Kristensson et al., 2004). Recently, it was shown that SMAC mimetic in conjunction to p38 MAPK inhibitor enhances caspase 8 activation in BMDMs (Lalaoui et al., 2016). We collected lysates from D5 and D12 BMDMs treated with BP with and without p38 MAPK inhibitor and performed western blot analysis for caspase 8. As expected, the cleaved form (active) of caspase 8 was detectable in D5 macrophages, and this was reduced in D12 macrophages (Fig. 16A). Interestingly, addition of the p38 MAPK inhibitor along with BP resulted in enhancement of caspase 8 activation in D12 macrophages (Fig. 16A). The densitometric analysis clearly revealed that in response to BP and p38 MAPK co-treatment, there was increase in level of cleaved (active) form of caspase 8 (p18) in D5 and D12 macrophages both (Fig. 16B, C), however, the increased activation of caspase 8 in D12 macrophages was more pronounced (Fig. 16C). These results indicate that the susceptibility to cell death induced in response to p38 MAPK inhibition in D12 macrophages is due to restoration of caspase 8 activation that was abrogated with prolonged differentiation of macrophages.

4.1.14. P38 MAPK signaling regulates ERK and JNK activation while has no impact on anti-apoptotic proteins XIAP and cFLIP$_L$

Since, MAP kinase-activated protein kinase 2 (MK2) has been previously reported to be one of the predominant target kinases downstream of p38 MAPK (Zarubin and Han, 2005), we evaluated the impact of inhibition of p38 MAPK on activation of MK2 by western blotting. We were also interested to see the impact of p38 MAPK inhibition on


A
Day 5
Day 12
BP
BP+p38i
BP
BP+p38i
Con
1h
3h
6h
1h
3h
6h
Con
1h
3h
6h
1h
3h
6h
50
20
37
Pro-Casp-8
Active Casp-8
β-actin

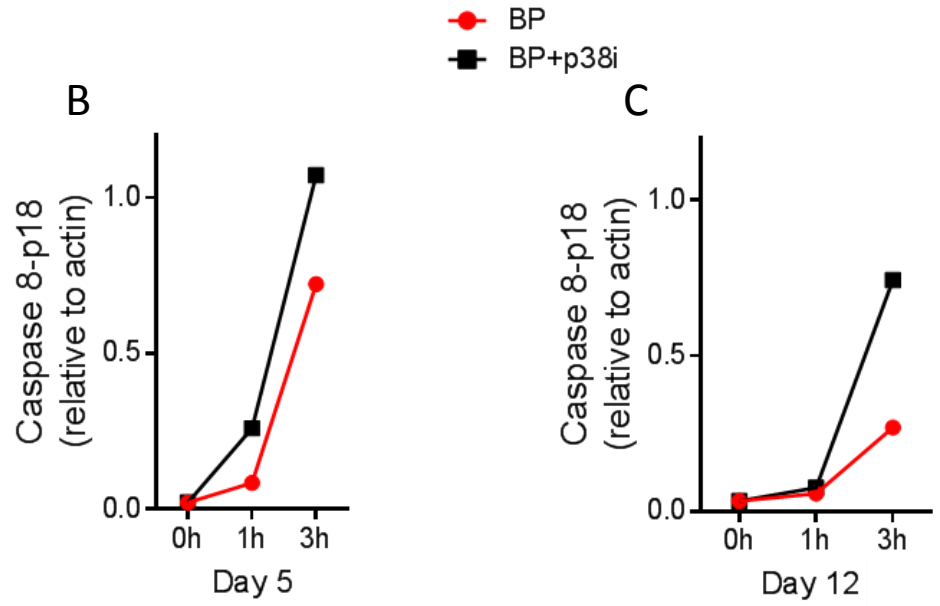

BP
BP+p38i
B
C
Caspase 8-p18
(relative to actin)
1.0
0.5
0.0
0h
1h
3h
Day 5
Day 12

Figure 16. **BP in conjunction to p38 MAPK inhibitor restores the activation of caspase 8 in D12 macrophages**. Lysates were collected from BP (10μM) +/- p38 MAPK inhibitor (5μM) treated WT macrophages over the course of 6 hours (as indicated), on day 5 and day 12 of differentiation to measure caspase 8 and β-actin by western blot (A). The buffer used for lysing was SDS buffer. Densitometry ratios of active caspase 8 (p18) post BP+/- p38 MAPK inhibitor in D5 (B) and D12 (C) macrophages were calculated by normalising to actin. The densitometric analysis is shown only for the first three-hour post treatment for each protein. All western blot experiments were done at least three times in three different mice.

JNK and ERK, the members of MAP kinase family important in the innate immune response (Strniskov et al., 2002). Recently it was reported that the deficiency in p38 MAPK or MK2 results in enhanced phosphorylation of JNK1/2 and ERK1/2 in BMDMs after SMAC mimetic treatment (Lalaoui et al., 2016). Since we observed enhanced activation of caspase 8 by p38 MAPK inhibitor (LY2228820) in our previous results, we wanted to see if enhanced caspase 8 activation was due to reduction in the levels of the anti-apoptotic proteins XIAP and $cFLIP_L$. Thus we evaluated all these proteins by western blotting. We collected lysates from BMDMs treated with BP with and without p38 MAPK inhibitor on D5 and D12 macrophages and performed western blot analysis for phosphorylated MK2, total MK2, XIAP, $cFLIP_L$, phosphorylated JNK, total JNK, phosphorylated ERK, total ERK and β-actin. BP treatment resulted in phosphorylation of MK2, and this effect was more pronounced for D5 macrophages, rather than for D12 macrophages (Fig. 17A). Inhibition of p38 MAPK resulted in abrogation of MK2 phosphorylation (Fig. 17A). The densitometric analysis further revealed the curtailment of MK2 phosphorylation with p38 MAPK inhibition in BP treated cells (Fig. 17B, C). The XIAP and the $cFLIP_L$ levels were not impacted by p38 MAPK inhibition as shown by the western blot and densitometric analysis (Fig. 17A, E, G).

The phosphorylation of JNK and ERK was higher in D12 macrophages compared to D5 macrophages after treatment with BP (Fig. 18A). Interestingly, addition of the p38 MAPK inhibitor along with BP resulted in increased phosphorylation of JNK and ERK in D5 and D12 macrophages (Fig. 18A). The densitometric analysis of pERK/ERK and pJNK/JNK clearly revealed the increased phosphorylation of JNK and ERK with p38 inhibition in cells treated with BP (Fig. 18C, E). Since the levels of phosphorylated ERK and JNK were higher in D12 macrophages, we treated the D5 and D12 WT BMDMs with BP with and without

ERK inhibitor and JNK inhibitor. Inhibition of ERK and JNK with specific inhibitors resulted in a modest increase in cell death in D5 macrophages; however, the inhibition of both the ERK and JNK MAPKs didn't restore the susceptibility of D12 macrophages to cell death (Fig. 18F, G). Taken together, these data indicate that p38 MAPK induces the phosphorylation of the downstream kinase MK2 and this signaling doesn't seem to impact the expression of the anti-apoptotic proteins XIAP and $cFLIP_{L.}$ Our data also indicated that p38 MAPK regulates the phosphorylation of ERK and JNK and although the prolonged differentiation of macrophages enhances the phosphorylation of both the MAPKs, they are not responsible for resistance of D12 macrophages to cell death.

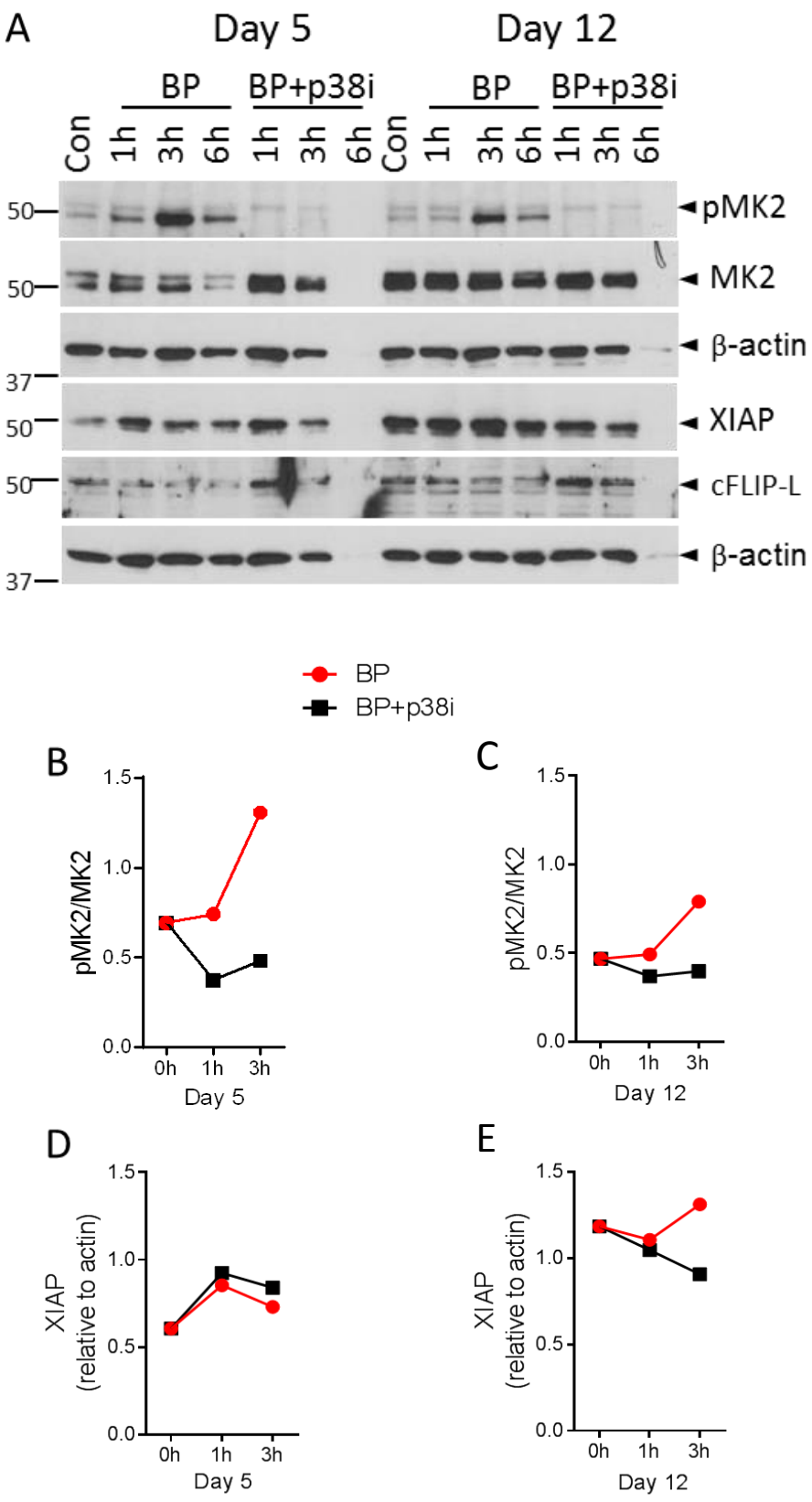

A
Day 5
Day 12
BP
BP+p38i
Con
1h
3h
6h
50
37
pMK2
MK2
β-actin
XIAP
cFLIP-L
BP
BP+p38i
B
pMK2/MK2
1.5
1.0
0.5
0.0
0h
1h
3h
Day 5
C
Day 12
D
XIAP
(relative to actin)
E

Figure 17. P38 MAPK inhibitor has no impact on the expression of XIAP and cFLIP$_L$. Lysates were collected from BP (10μM) +/- p38 MAPK inhibitor (5μM) treated WT macrophages over the course of 6 hours, on day 5 and day 12 of differentiation to measure pMK2, MK2, XIAP, cFLIP$_L$ and β-actin by western blot (A). Densitometry ratios of pMK2/MK2 were calculated in response to BP with and without p38 MAPK inhibitor (B, C). Densitometry of XIAP and cFLIP$_L$ were calculated by normalising to actin with BP treatment (D, F) and with BP+p38 MAPK inhibitor treatment (E, G). All western blot experiments were done at least three times in three different mice.

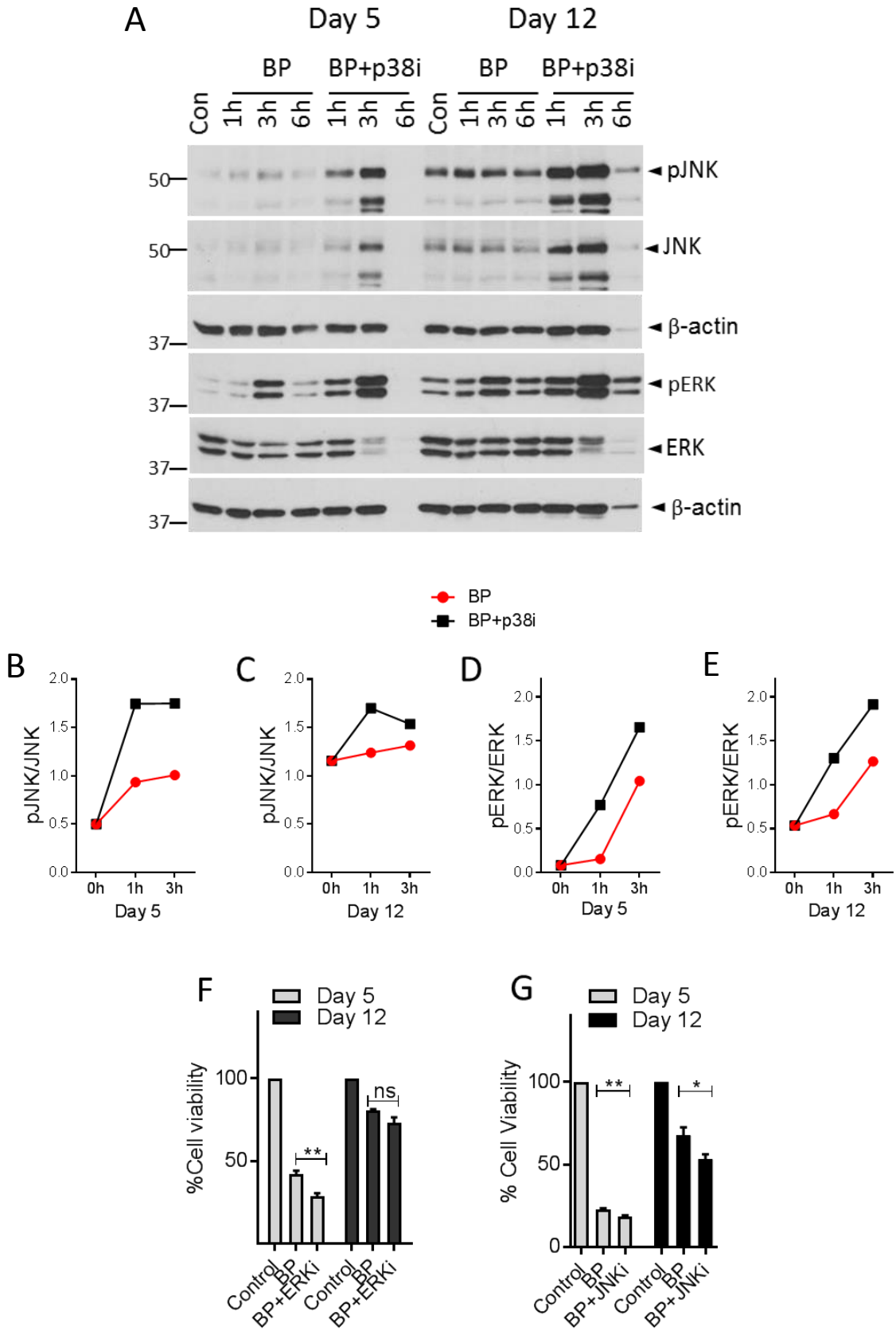

A
Day 5
Day 12
BP
BP+p38i
Con
1h
3h
6h
50
37
pJNK
JNK
β-actin
pERK
ERK
β-actin
BP
BP+p38i
B
C
D
E
pJNK/JNK
pERK/ERK
0h
1h
3h
Day 5
Day 12
F
G
Day 5
Day 12
%Cell viability
% Cell Viability
100
50
0
**
ns
*
Control
BP
BP+ERKi
BP+JNKi

Figure 18. P38 MAPK regulates the activation of JNK and ERK. Lysates were collected from BP (10μM) +/- p38 MAPK inhibitor (5μM) treated WT macrophages over the course of 6 hours, on day 5 and day 12 of differentiation to measure pJNK, JNK, pERK, ERK and β-actin by western blot (A). Densitometry ratios of pJNK/JNK and pERK/ERK were calculated with BP treatment (B, D) and with BP+p38 MAPK inhibitor treatment (C, E). WT BMDMS were treated with BP+/- ERKi (F) and JNKi (G) and cell viability measured by MTT assay. All western blot experiments and cell viability assays were done at least three times in three different mice.

4.1.15. MK2 regulates BP induced caspase 8 activation, TNF-α production and cell death.

In our previous results we observed that treatment of cells with the p38 MAPK inhibitor resulted in inhibition of phosphorylation of MK2. We therefore wanted to determine whether MK2 is the downstream target kinase of p38 MAPK that regulates BP induced cell death of macrophages. BMDMs were generated from WT and MK2-/- mice at day 9 of differentiation (We used D9 macrophages due to increasing death of macrophages due to some unknown reasons in the petri dish) and treated with BP. Nec-1 was added to some cells to evaluate the mechanism of cell death. MK2-/- mouse have dysfunctional MK2 kinase activity due to mutation in MK2 gene (Kotlyarov et al., 1999). As expected, D9 WT macrophages were resistant to cell death in response to BP treatment (Fig. 19A). Interestingly, the resistance of macrophages to cell death in response BP treatment was abrogated in MK2-/- macrophages (Fig. 19A). Cell death was rescued by Nec-1, indicating ripoptosome signaling as the inducer of cell death (Fig. 19A). We collected lysates from WT and MK2-/- macrophages after treatment with BP, and performed western blot analysis for caspase 8, XIAP, RipK1, pMK2, MK2 and β-actin. As expected, the cleaved form (active) of caspase 8 was not detected in WT macrophages but was detected in MK2-/- macrophages (Fig. 19B). This suggests that MK2 regulates the activation of caspase 8. XIAP levels were not impacted by MK2 inactivation in macrophages (Fig. 19C). We observed the minimal phosphorylation of RipK1 in D9 macrophages but that was completely abolished in macrophages with an inactive MK2, suggesting that MK2 is the kinase that phosphorylates RipK1 during ripoptosome signaling (Fig. 19C, D). We also observed that the phosphorylation of MK2 was abrogated in MK2-inactive macrophages (Fig. 19C). We also

evaluated the TNF-α expression by bioassay on WEHI cells in WT and MK2-/- macrophages at day 9 of differentiation. Interestingly, there was significantly higher level of TNF-α expression in MK2-/- macrophages compared to WT macrophages (Fig. 19F).

These data indicate that MK2 is activated downstream of p38 MAPK signaling and despite the low level activation of these kinases in D12 macrophages, they are highly functional in regulating caspase 8 activation and TNF-α expression. Furthermore, our results appear to indicate that the impairment in RipK1 phosphorylation in D12 macrophages in response to BP treatment is due to lower levels of phosphorylated MK2.

4.1.16. XIAP regulates phosphorylation of MK2 during BP treatment of macrophages.

Previously, we have shown that deficiency of XIAP in D12 macrophages restores the activation of caspase 8 following BP treatment. We were interested in determining whether XIAP deficiency impacts the phosphorylation of MK2, RipK1 and $cFLIP_L$ in differentiated macrophages. We collected lysates from BP treated D12 BMDMs of WT and XIAP-/- mice, and performed western blot analysis for pMK2, MK2, RipK1, $cFLIP_L$ and β-actin. We observed that treatment of macrophages with BP resulted in a greater increase in the phosphorylation of MK2 in XIAP-/- macrophages (Fig. 20A). This was confirmed by performing densitometric analysis of pMK2/MK2 in WT and XIAP-/- macrophages (Fig. 20B). Phosphorylation of RipK1 and the expression of $cFLIP_L$ were not impacted by XIAP deficiency (Fig. 20A, C, and D). These data indicate that XIAP seem to regulate the phosphorylation (activation) of MK2, which can be correlated with the resistance to cell death developed following prolonged differentiation of macrophages with BP treatment.

Day 9 Macrophages

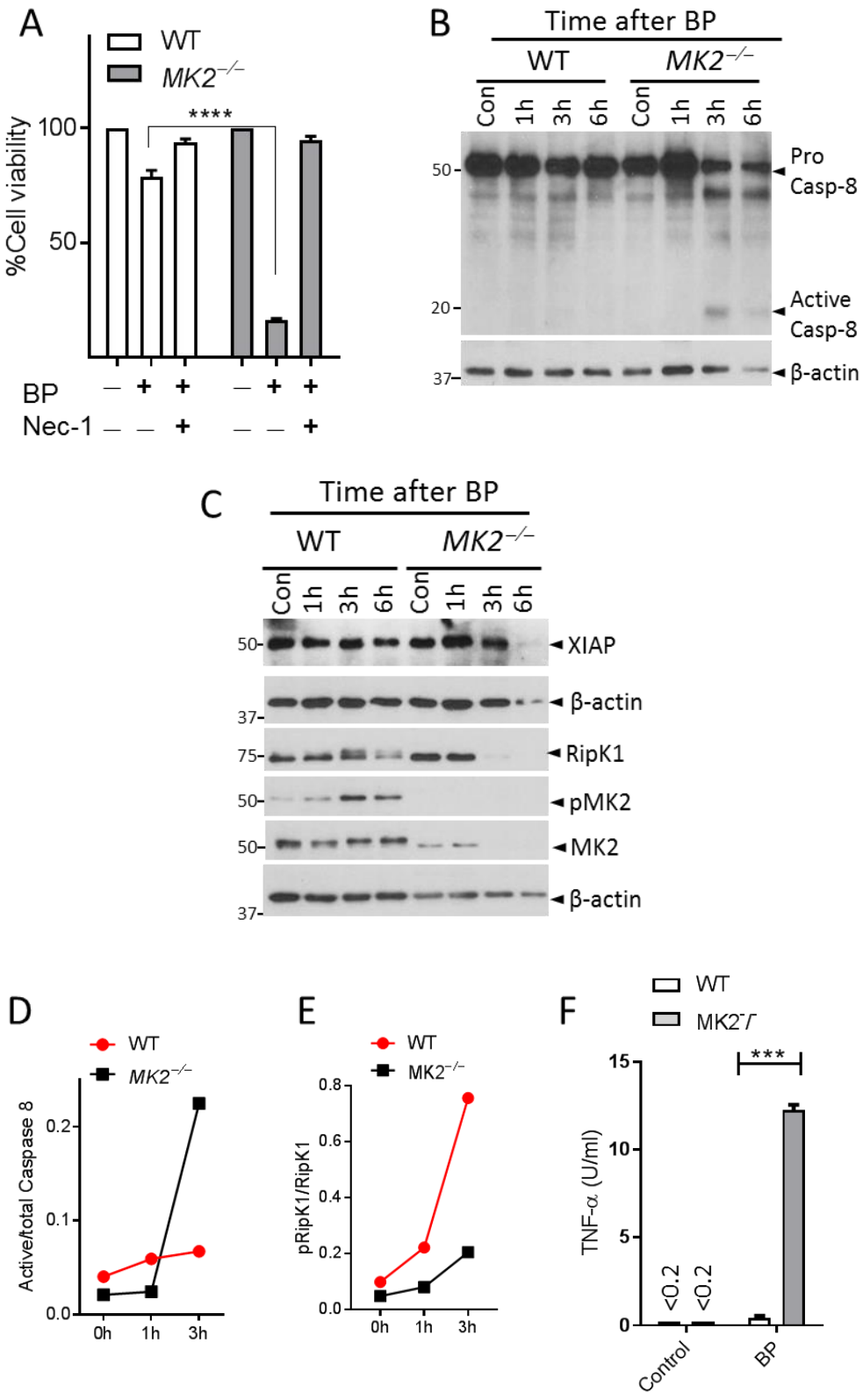


Figure 19. MK2 deficiency activates caspase 8 and TNF-α expression and impairs RipK1 phosphorylation. BMDM*s* from WT (C57BL/6) and MK2 -/- mice isolated at day 9 of differentiation were treated with BP (10μM) with and without Nec-1 (10μM). After 24 hours incubation, the cell viability was measured by MTT assay (A). Lysates were collected from BP (10μM) treated macrophages over the course of 6 hours (as indicated) to measure caspase 8 (B), XIAP, RipK1, pMK2, MK2 and β-actin (B) by western blot. The buffer used for lysing was SDS buffer. Densitometry ratios of p18 (activated caspase 8)/actin (D) and pRipK1/RipK1 (E) were calculated in response to BP treatment. The densitometric analysis is shown only for the first three-hour post treatment for each protein. The supernatants of WT and MK2-/- macrophages treated with BP (10μM) for 24 hours were assessed for TNF-α production using the bioassay on WEHI cells (F). The assay detects the cytotoxic effect of the TNF-α, present in the supernatant, on WEHI cells and the cell viability is measured by MTT assay. The cell death is directly proportional to the TNF-α present in the supernatant. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

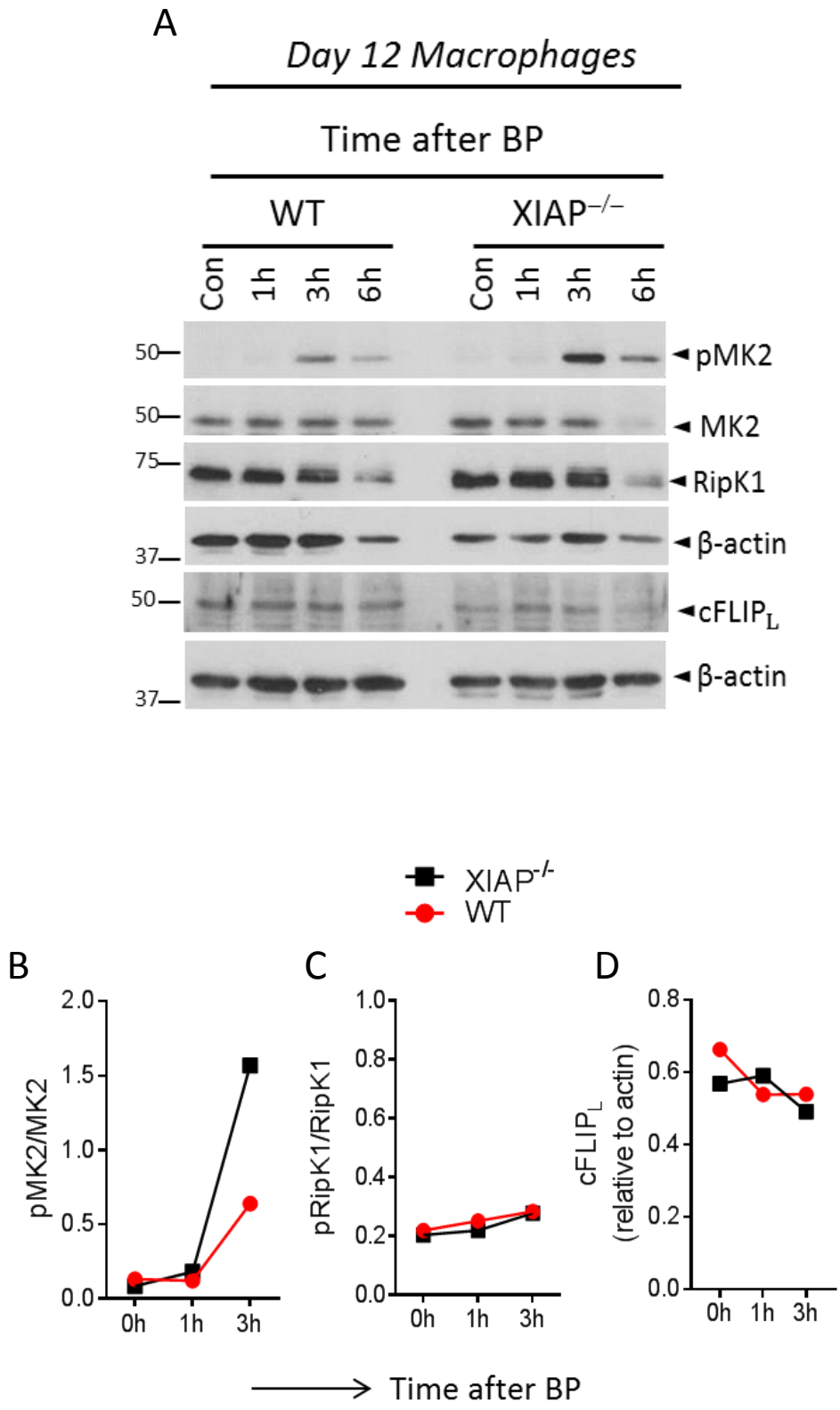

A
Day 12 Macrophages
Time after BP
WT
XIAP−/−
Con
1h
3h
6h
Con
1h
3h
6h
50
pMK2
50
MK2
75
RipK1
37
β-actin
50
cFLIPL
37
β-actin
XIAP-/-
WT
B
pMK2/MK2
2.0
1.5
1.0
0.5
0.0
0h
1h
3h
C
pRipK1/RipK1
1.0
0.8
0.6
0.4
0.2
0.0
0h
1h
3h
D
cFLIPL (relative to actin)
0.8
0.6
0.4
0.2
0.0
0h
1h
3h
Time after BP

Figure 20. XIAP regulates MK2 activation. Lysates were collected from WT and XIAP-/- macrophages isolated at day 12 of differentiation treated with BP (10μM) over the course of 6 hours (as indicated) to measure pMK2. MK2, RipK1, $cFLIP_L$ and β-actin by western blot (A). The buffer used for lysing was SDS buffer. Densitometry ratios of pMK2/MK2 (B), pRipK1/RipK1 (C) and $cFLIP_L$ normalised to actin (D) were calculated in response to BP treatment. The densitometric analysis is shown only for the first three-hour post treatment for each protein. All western blot experiments were done at least three times in three different mice.

4.1.17. TNF-α promotes phosphorylation of RipK1 and p38 MAPK in response to BP treatment.

Our previous results suggest that TNF-α signaling is a crucial and indispensable mechanism of BP induced cell death of macrophages. Since, phosphorylation of RipK1, p38 MAPK and MK2 was impaired in D12 macrophages, we were interested in evaluating the impact of TNF-α expression on the phosphorylation of these kinases during ripoptosome signaling. We collected lysates from BP treated BMDMs of WT and TNF-α-/- mice at day 5 of differentiation and performed western blot analysis for RipK1, pMK2, MK2, phospho p38 (pP38 MAPK), p38 and β-actin. Interestingly, RipK1 phosphorylation was detected in WT macrophages but abrogated in TNF-α-/- macrophages (Fig. 21A). Similarly, the phosphorylation of p38 MAPK and MK2 were detected at higher levels in WT macrophages and this was diminished in TNF-α-/- macrophages (Fig. 21A). Densitometric analysis revealed the reduction in phosphorylation of RipK1 (Fig. 21 B) and MK2 (Fig. 21C) in TNF-α-deficient macrophages. These results indicate that TNF-α signaling promotes phosphorylation of RipK1, p38 MAPK and MK2, which promotes ripoptosome-induced cell death in D5 macrophages.

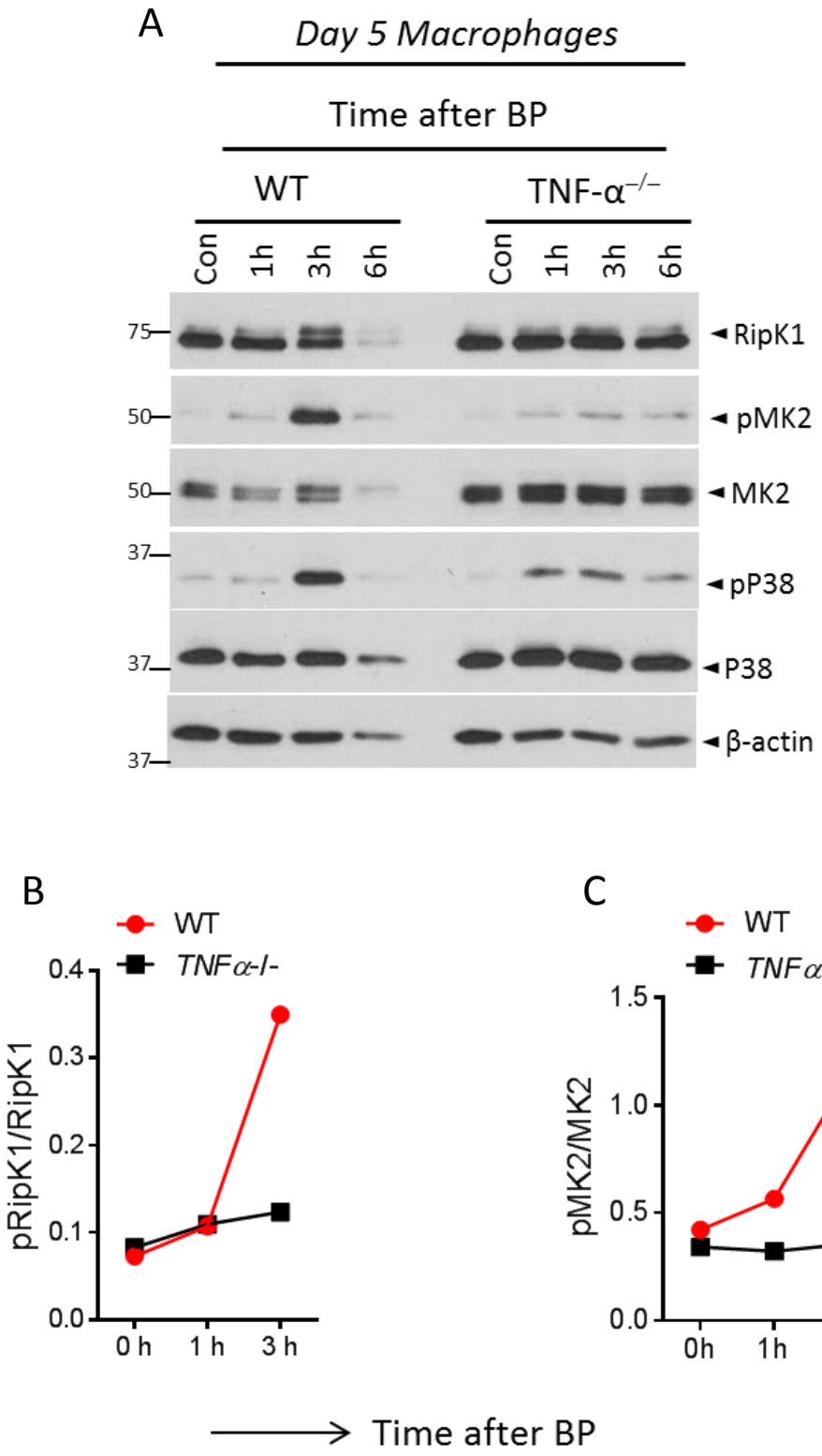

A
Day 5 Macrophages
Time after BP
WT
TNF-α−/−
Con
1h
3h
6h
Con
1h
3h
6h
75
50
50
37
37
37
RipK1
pMK2
MK2
pP38
P38
β-actin
B
WT
TNFα-I-
pRipK1/RipK1
0.4
0.3
0.2
0.1
0.0
0 h
1 h
3 h
C
WT
TNFα-/-
pMK2/MK2
1.5
1.0
0.5
0.0
0h
1h
3h
Time after BP

Figure 21. Phosphorylation of RipK1, p38 and MK2 is abrogated in the absence of TNF-α. Lysates were collected from WT and TNF-α-/- macrophages isolated at day 5 of differentiation treated with BP (10μM) over the course of 6 hours (as indicated) to measure RipK1, pMK2. MK2, pP38 MAPK, total p38 MAPK and β-actin by western blot (A). The buffer used for lysing was SDS buffer. Densitometry ratios of pRipK1/RipK1 (B) and pMK2/MK2 (C) were calculated in response to BP. The densitometric analysis is shown only for the first three-hour post treatment for each protein. All western blot experiments were done at least three times in three different mice.

4.2. Concurrent TLR activation doesn't rescue BP-induced cell death of differentiated macrophages

4.2.1. Stimulation of macrophages by LPS does not overcome the resistance of macrophages induced by BP

We wished to determine whether concomitant engagement of TLR signaling would influence BP induced cell death of macrophages. We purified monocytes, and isolated macrophages at day 5 (D5) and day 12 (D12) of differentiation with M-CSF, and treated them with the TLR4 ligand LPS with and without BP. Nec-1 was added to some cells to evaluate the mechanism of cell death. Treatment of monocytes with LPS did not rescue cell death induced by BP (Fig. 22A). Interestingly, cell death with LPS+BP was only partially inhibited by Nec-1 (Fig. 22A), which is in contrast to cells treated with BP only where Nec-1 treatment resulted in complete rescue of cell death (Fig. 5A). Similar to monocytes, D5 BMDMs were highly susceptible to cell death by LPS+BP treatment (Fig. 22B), and interestingly Nec-1 failed to inhibit cell death (Fig. 22B). Failure of Nec-1 to rescue LPS+BP induced cell death indicates that the mechanism of cell death may be different. D12 macrophages were highly resistant to LPS+BP induced cell death (Fig. 22B). Since we had previously observed that BP induced cell death is detectable only after 6 hours post treatment (Fig. 5C), we evaluated cell death at 6h and 9h post LPS+BP treatment, and observed that cell death was not significant at 6 hour but was highly significant at 9 hours of LPS+BP treatment (Fig. 22C). These results suggest that cell death induced by BP or LPS+BP treatment operates at a slower speed. The cell death in the context of TLR4 signaling was also tested using a different SM, BV6 and we got similar results as with BP treatment

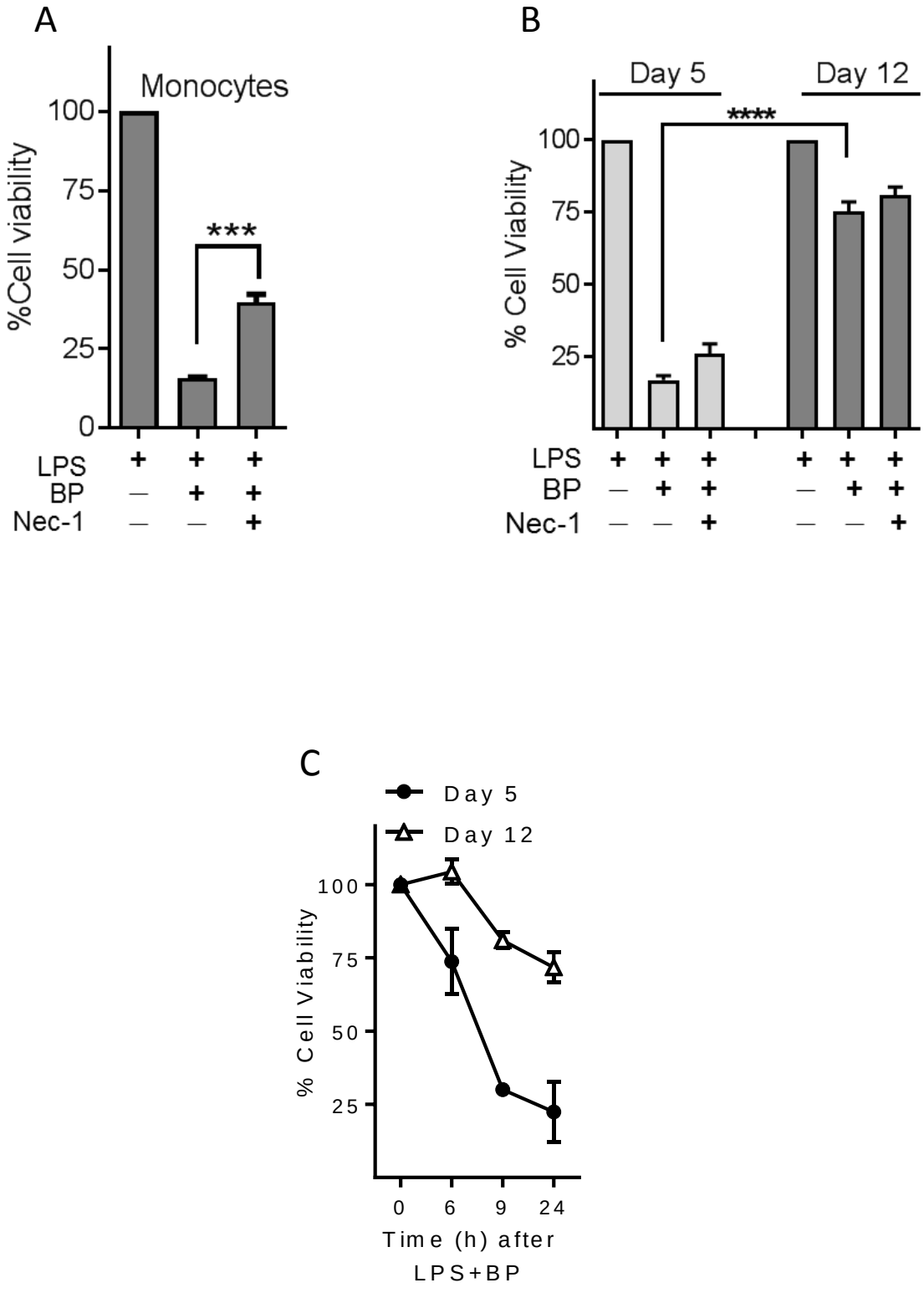

A
Monocytes
%Cell viability
100
75
50
25
0
***
LPS + + +
BP — + +
Nec-1 — — +
B
Day 5
Day 12
****
% Cell Viability
100
75
50
25
LPS + + + + + +
BP — + + — + +
Nec-1 — — + — — +
C
Day 5
Day 12
% Cell Viability
100
75
50
25
0 6 9 24
Time (h) after
LPS+BP

Figure 22. Macrophages develop resistance to LPS+BP induced cell death as they differentiate. Monocytes and D5 and D12 BMDMs from WT (C57BL/6) mice were treated with LPS (1ng/ml), BP (10µM) and Nec-1 (10µM) as indicated (A, B). After 24 hours incubation, the cell viability was measured by MTT assay. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

(Appendix-Fig. III). Thus, these results indicate that activation of TLR4 signaling doesn't rescue the cell death induced by SMAC mimetic, but appears to switch the mode of cell death to another pathway that is not dependent on the kinase activity of RipK1. Furthermore, our results indicated that the prolonged differentiation of macrophages results in acquisition of resistance to this pathway of cell death as well.

4.2.2. Cell death induced by LPS+BP is partially dependent on TNF-α/TNF-R1 and RipK1 signaling while significantly dependent on RipK3 signaling

Since Nec-1 failed to induce complete rescue of cell death induced by LPS+BP, we wished to investigate the mechanism of cell death further, and investigated the role of TNF-α/TNF-R signaling in the context of LPS+BP induced cell death in macrophages. We also evaluated cell death in RipK3-/-, and the kinase inactive RipK1^{K45A} macrophages following treatment with LPS+BP. Unlike BP induced cell death that was completely dependent on TNF-R1 signaling (Fig. 6A), cell death induced by LPS+BP treatment occurred through a mechanism that was modestly dependent on TNF-R1 (Fig. 23A). TNF-R2 deficiency also had no impact on LPS+BP induced cell death (Fig. 23A). Furthermore, macrophages that were deficient in TNF-α-/- are partially resistant to LPS+BP induced cell death (Fig. 23B), in contrast to cell death induced by BP that was completely dependent on TNF-α (Fig. 6B). While RipK1^{K45A} macrophages showed partial resistance to cell death induced by LPS+BP (Fig. 23C), RipK3-/- macrophages showed enhanced resistance to cell death compared to WT macrophages (Fig. 23D). We further investigated the role of the kinase activity of RipK3 in LPS+BP induced cell death by treating monocytes and D5 and D12 BMDMs with LPS+BP in the presence or absence of GSK 872 (a RipK3 kinase inhibitor). GSK 872 didn't

rescue the cell death following LPS+BP treatment (data not shown). LPS+BP induced cell death was not dependent on IFNAR1, MyD88 or TRIF signaling (Appendix-Fig. IVa, b, c). Taken together, these results indicate concurrent treatment of cells with LPS+BP results in cell death of macrophages that is distinct from the typical ripoptosome signaling mechanism, that is partially dependent on the RipK1 kinase activity (in contrast to BP induced cell death). Furthermore, RipK3 promotes LPS+BP induced cell death in a manner that is independent of the kinase function of RipK3 indicating that the role of RipK3 in BP or LPS+BP induced cell death remains unchanged.

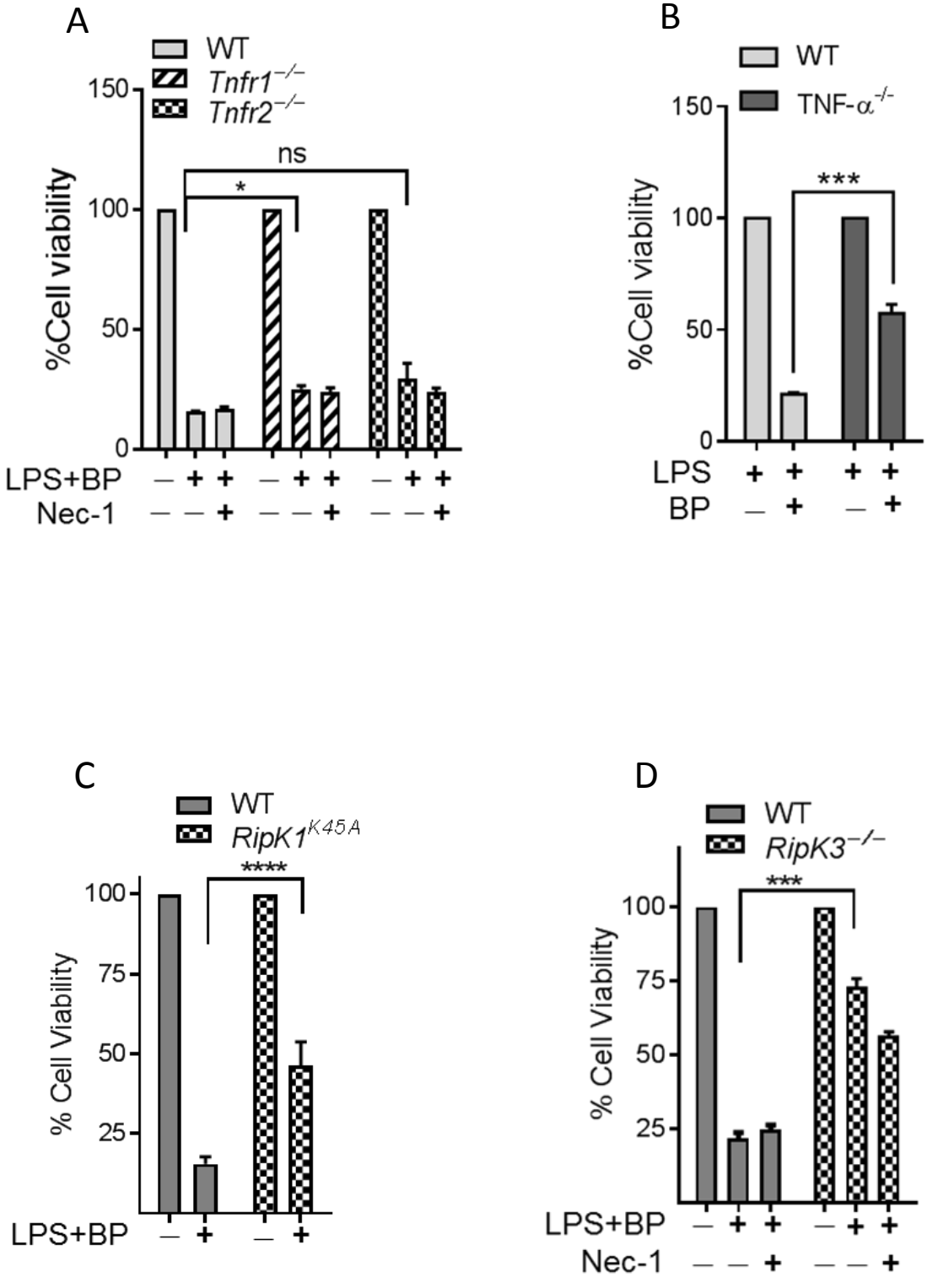

A
WT
Tnfr1−/−
Tnfr2−/−
%Cell viability
150
100
50
0
ns
*
LPS+BP
Nec-1
B
WT
TNF-α-/-
%Cell viability
***
LPS
BP
C
WT
RipK1K45A
****
% Cell Viability
100
75
50
25
LPS+BP
D
WT
RipK3−/−
***
% Cell Viability
LPS+BP
Nec-1

Figure 23. LPS+BP induced cell death is dependent on RipK3 scaffold and partially dependent on TNF-α and RipK1 signaling. BMDMs from WT (C57BL/6), TNF-R1-/-, TNF-R2-/-, TNF-α-/-, RipK1^{K45A} and RipK3-/- mice (day 5 of growth) were treated with LPS (1ng/ml), BP (10μM) and Nec-1 (10μM) as indicated. After 24 hours incubation, the cell viability was measured by MTT assay. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

4.2.3. Addition of LPS in differentiated macrophages restores phosphorylation of RipK1 during treatment with BP.

We deciphered the mechanism behind the resistance of macrophages of cell death induced by LPS+BP, and evaluated the impact on the activation of RipK1 and RipK3 by western blotting. We collected cell lysates from D5 and D12 WT BMDMs following treatment with LPS with and without BP for varying time intervals. Following LPS or LPS+BP treatment, phosphorylation of RipK1 was detected at 1hour post stimulation in D5 macrophages and this activation of RipK1 was detectable in D12 macrophages as well (Fig. 24A). The densitometric analysis of pRipK1/RipK1 revealed similar phosphorylation of RipK1 in D5 and D12 macrophages following LPS or LPS+BP treatment (Fig 24B, D). These results are in contrast to our previous results where we observed that D12 macrophages fail to phosphorylate RipK1 following treatment with BP (Fig. 8A). To determine whether the mode of cell death following LPS+BP treatment was switched to necroptosis, we evaluated the phosphorylation of RipK3 by western blotting, and observed no phosphorylation of RipK3 in D5 or D12 macrophages (Fig. 24A). Densitometric analysis was done for pRipK3/RipK3 in macrophages at both stages of differentiation following LPS and LPS+BP treatment (Fig. 24C, E). These results suggest that LPS+BP does not induce cell death by necroptosis. Taken together, these data indicate that impairment in RipK1 phosphorylation observed in D12 macrophages in the context of ripoptosome signaling is restored with the addition of LPS and the concurrent treatment of cells with LPS+BP doesn't seem to switch the mechanism of cell death to necroptosis

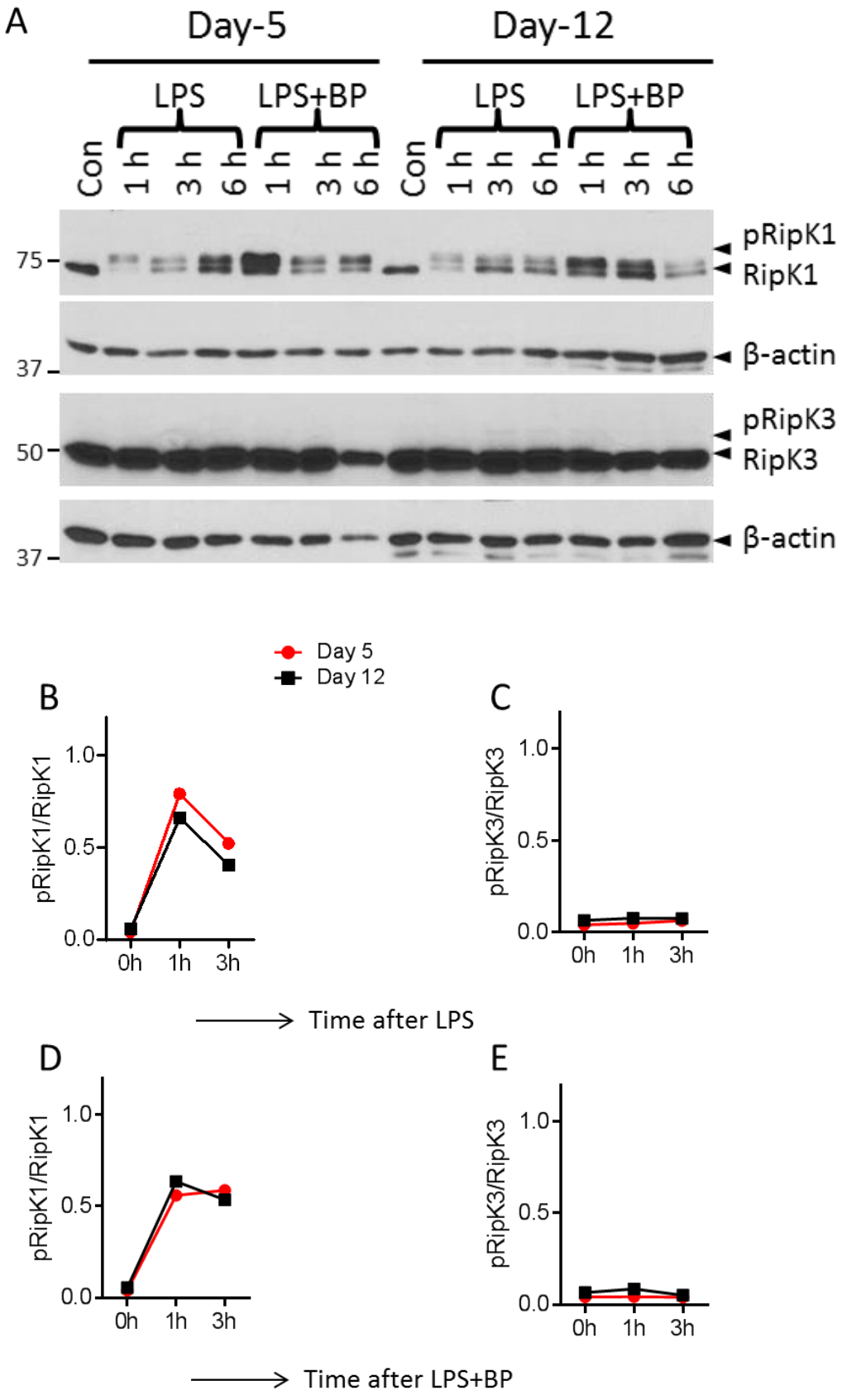

A
Day-5
Day-12
LPS
LPS+BP
LPS
LPS+BP
Con
1 h
3 h
6 h
1 h
3 h
6 h
Con
1 h
3 h
6 h
1 h
3 h
6 h
75
37
50
37
pRipK1
RipK1
β-actin
pRipK3
RipK3
β-actin
Day 5
Day 12
B
C
pRipK1/RipK1
pRipK3/RipK3
1.0
0.5
0.0
0h
1h
3h
Time after LPS
D
E
pRipK1/RipK1
pRipK3/RipK3
1.0
0.5
0.0
0h
1h
3h
Time after LPS+BP

Figure 24. RipK1 phosphorylation is not impaired by LPS+BP treatment in differentiated macrophages. Lysates were collected from LPS (1ng/ml) +/- BP (10μM) treated WT macrophages over the course of 6 hours, on day 5 and day 12 of differentiation to measure RipK1, RipK3 and β-actin by western blot (A). The buffer used for lysing was SDS buffer. Densitometric ratios of phosphorylated RipK1 to unphosphorylated RipK1 (pRipK1/RipK1), pRipK3/RipK3 following LPS (B, C) and LPS+BP (D, E) treatment were calculated. The densitometric analysis is shown only for the first three-hour post treatment for each protein. All western blot experiments were done at least three times in three different mice.

4.2.4. Resistance of highly differentiated macrophages to cell death by LPS+BP correlates to high levels of XIAP and cFLIP$_L$ and is abrogated in XIAP-deficient cells

Having observed that addition of LPS in differentiated macrophages restores phosphorylation of RipK1 during treatment with BP, we wished to evaluate the impact of LPS+BP on the expression levels of anti-apoptotic proteins (IAPs) and NF-kB by western blotting. We collected cell lysates from D5 and D12 WT BMDMs following treatment with LPS+BP for varying time intervals. The expression levels of XIAP and cFLIP$_L$ was high in D12 macrophages, and this was not reversed by co-treatment of cells with LPS (Fig. 25A). Interestingly, degradation of cIAPs which occurs following BP treatment, was not impacted by concurrent treatment with LPS (Fig. 25A). Since LPS is a potent activator of NF-kB pathway, we detected the phosphorylated p65 levels of macrophages at both stages of differentiation following LPS+BP treatment. In response to LPS+BP treatment the expression of phosphorylated p65 was increased in D5 macrophages and this activation was slightly reduced in D12 macrophages (Fig. 25A). The densitometric analysis was done for the expression levels of these proteins following LPS+BP treatment (Fig. 25B, C, D). Since we observed elevation of XIAP levels in D12 macrophages, we thus wished to determine whether XIAP deficiency has an impact on the cell death resistance developed by the differentiated cells in response to LPS+BP treatment like BP treatment. D5 and D12 BMDMs were generated from WT and XIAP-/- mice and treated with LPS+BP. The cell viability was assessed by MTT assay. There was enhancement of cell death in D5 XIAP-/- macrophages compared to D5 WT macrophages (Fig. 25E). Interestingly, the resistance of D12 macrophages following LPS+BP treatment was abrogated in XIAP-/- macrophages (Fig. 25E). Together, these results indicate that that the expression levels of anti-apoptotic

proteins in BP or LPS+BP induced cell death remained high in D12 macrophages and LPS+BP induced cell death is also regulated by XIAP in highly differentiated macrophages.

4.2.5. Treatment of D12 macrophages with LPS does not restore the processing of caspase 8 following BP treatment

Previous results indicated that the resistance of D12 macrophages to cell death correlate with the impaired activation of caspase 8. We wished to determine whether co-treatment of cells with LPS would restore the processing of caspase 8 in D12 macrophages. We collected the lysates from D5 and D12 WT BMDMs treated with BP or LPS+BP and performed western blot analysis of caspase 8. The cleaved form (active) of caspase 8 was detectable in D5 macrophages following BP and LPS+BP treatment and this was substantially reduced in D12 macrophages (Fig. 26A). We also measured the activity of caspase 8, caspase 9 and caspase 3/7 by luminescence bioassays in D5 and D12 macrophages following LPS+BP treatment. D5 cells treated with LPS+BP showed high caspase 8, caspase 9 and caspase 3/7 activity in comparison to D12 cells (Fig. 26B, C, D). Addition of the pan-caspase inhibitor zVAD (Fig. 26A and D) and caspase 9 inhibitor Z-LEHD (Fig. 26C) reduced the activity of all three caspases to basal levels. These results suggest that the concurrent addition of LPS with BP doesn't restore the processing of caspase 8 in D12 macrophages. Taken together these results indicate that LPS treatment doesn't restore the activation of caspase 8 in D12 macrophages following BP treatment and the cell death induced by LPS+BP can be correlated to the increased activation of caspase 8, 9 and 3/7 which is impaired in macrophages that undergo prolonged differentiation.

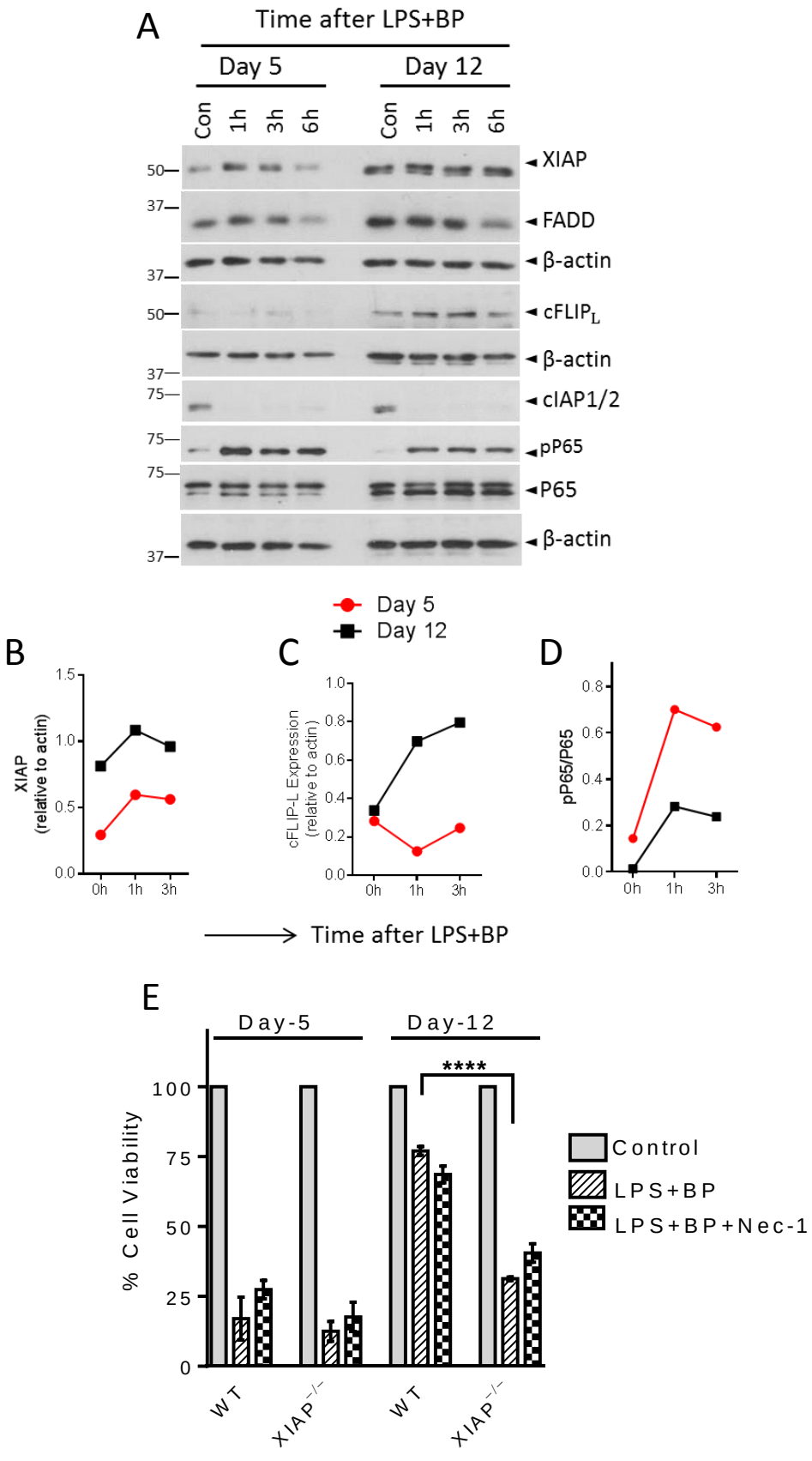
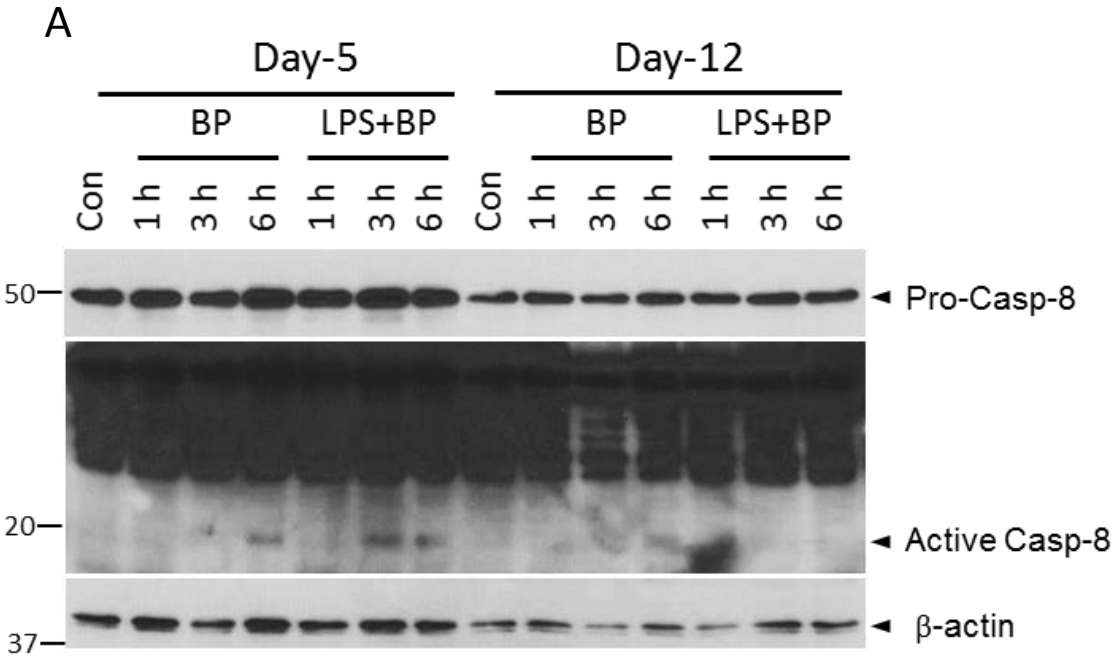

A
Time after LPS+BP
Day 5
Day 12
Con
1h
3h
6h
XIAP
FADD
β-actin
cFLIP_L
β-actin
cIAP1/2
pP65
P65
β-actin
50
37
37
50
37
75
75
75
37
B
C
D
Day 5
Day 12
XIAP (relative to actin)
cFLIP-L Expression (relative to actin)
pP65/P65
0h
1h
3h
Time after LPS+BP
E
Day-5
Day-12
****
% Cell Viability
100
75
50
25
0
WT
XIAP-/-
WT
XIAP-/-
Control
LPS+BP
LPS+BP+Nec-1

Figure 25. Elevated levels of XIAP and cFLIP$_L$ correlates to cell death resistance in D12 macrophages post LPS+BP treatment. Lysates were collected from LPS (1ng/ml) + BP (10μM) treated WT macrophages over the course of 6 hours (as indicated), on day 5 and day 12 of differentiation to measure XIAP, FADD, cFLIP$_L$, cIAPs, pp65, p65 and β-actin by western blot (A). The buffer used for lysing was SDS buffer. Densitometric ratios of phosphorylated XIAP (B), cFLIP$_L$ (C) both normalised to actin and pp65/p65 (D) following LPS+BP treatment were calculated. BMDMs from WT (C57BL/6) and XIAP -/- mice were treated with LPS (1ng/ml) + BP (10μM) with and without Nec-1 (10μM). After 24 hours incubation, the cell viability was measured by MTT assay (E). Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.


A
Day-5
Day-12
BP
LPS+BP
BP
LPS+BP
Con
1 h
3 h
6 h
1 h
3 h
6 h
Con
1 h
3 h
6 h
1 h
3 h
6 h
50
20
37
Pro-Casp-8
Active Casp-8
β-actin

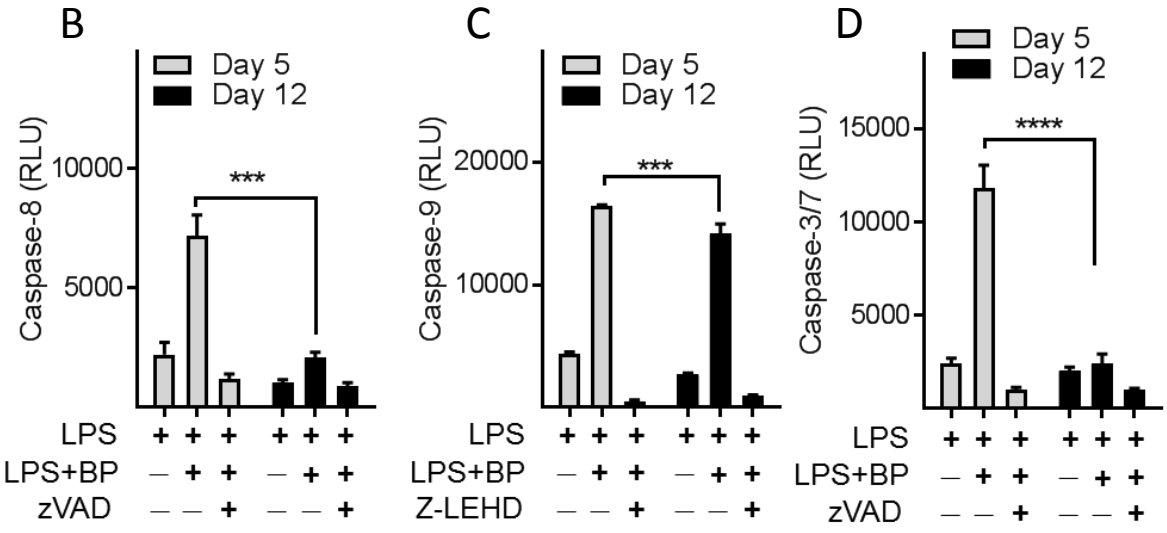
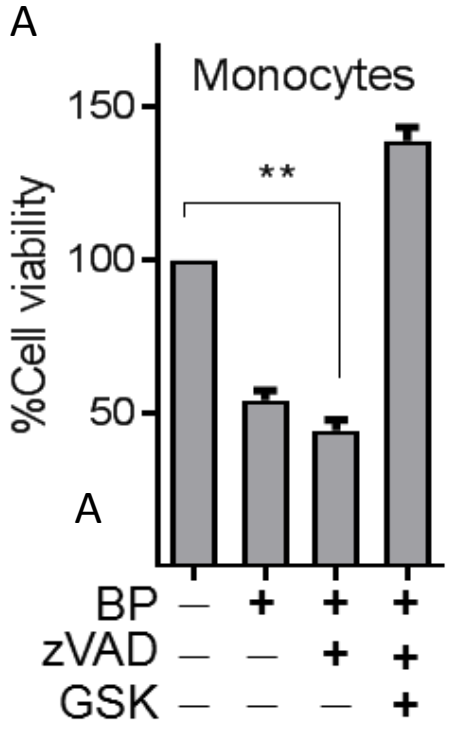

B
Caspase-8 (RLU)
10000
5000
Day 5
Day 12
***
LPS
LPS+BP
zVAD
C
Caspase-9 (RLU)
20000
10000
Day 5
Day 12
***
LPS
LPS+BP
Z-LEHD
D
Caspase-3/7 (RLU)
15000
10000
5000
Day 5
Day 12
****
LPS
LPS+BP
zVAD

Figure 26. Activation of caspase 8, 9 and 3/7 is impaired with prolonged differentiation of macrophages following treatment with LPS+BP. Lysates were collected from LPS (1ng/ml) + BP (10µM) treated WT macrophages over the course of 6 hours (as indicated), on day 5 and day 12 of differentiation to measure caspase 8 and β-actin by western blot (A). The buffer used for lysing was SDS buffer. Macrophages treated with BP (10µM) with and without zVAD (50µM) (B, D) Z-LEHD (50µM) (C) was assessed by Caspase-Glo® 8 (B), Caspase-Glo® 9 (C), Caspase- Glo® 3/7 (D) assays and the luminescence was measured. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

4.3. Impact of macrophage differentiation on susceptibility to necrosome signaling

4.3.1. Inhibition of caspases shifts BP induced cell death to necroptosis, which is not impacted by macrophage differentiation

Inhibition of caspases induces an alternative form of cell death called necroptosis that operates in the absence of caspase signaling and in the presence of the kinase activity of RipK1 and RipK3 (Degterev et al., 2005). It has been previously reported that treatment of cells with SMAC mimetic in conjunction with the caspase inhibitor zVAD, induces necroptosis (McComb et al., 2014). We wished to determine whether the prolonged differentiation of macrophages results in their resistance to cell death by necroptosis. We isolated macrophages at day 5 (D5) and day 12 (D12) of differentiation with M-CSF and treated them with BP+/-zVAD with and without GSK or Nec-1. We also evaluated the impact on purified monocytes from bone marrow. As expected, treatment of monocytes with BP induced cell death in monocytes (Fig. 27A). Addition of zVAD along with BP failed to rescue the cell death (Fig. 27A). Rather, zVAD treatment resulted in slightly enhanced cell death (Fig. 27A). Addition of GSK, which inhibits RipK3 kinase activity, in BP+zVAD treated cells resulted in complete rescue of the cell death, indicative of the mechanism of cell death to be necroptosis (Fig. 27A). D5 macrophages were highly susceptible to cell death induced by BP+zVAD (Fig. 27B). Addition of GSK resulted in complete rescue of the cell death induced by BP+zVAD (Fig. 27B). While D12 macrophages were resistant to BP induced cell death, they were highly susceptible to BP+zVAD induced cell death (Fig. 27B). These results indicate that inhibition of caspases results in RipK3-mediated necroptosis of monocytes and BMDMs following BP-treatment, and the prolonged differentiation of macrophages does not result in resistance to cell death by this pathway.


A
Monocytes
150
100
50
%Cell viability
**
A
BP — + + +
zVAD — — + +
GSK — — — +

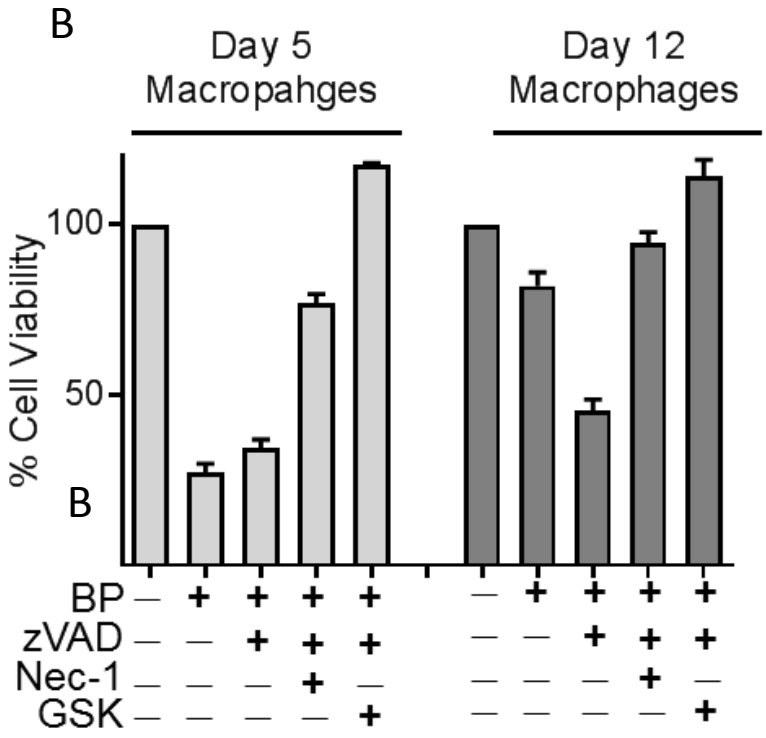
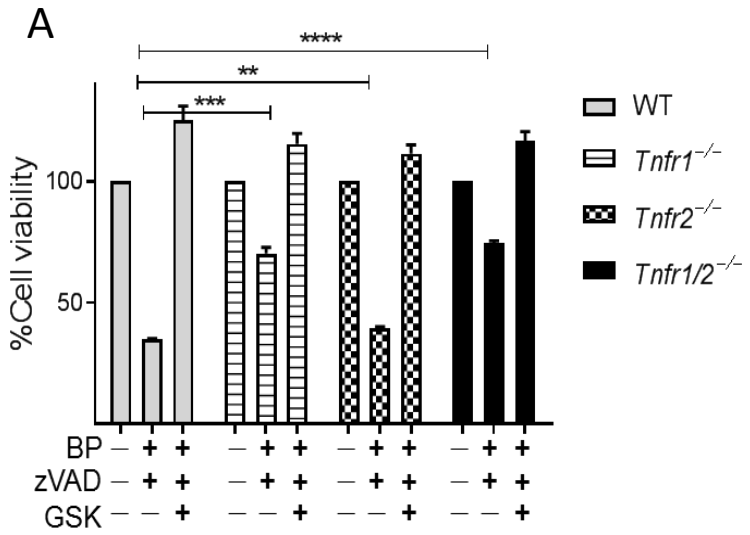
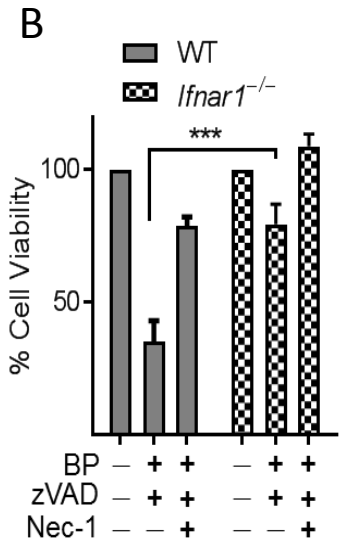

B
Day 5
Macropahges
Day 12
Macrophages
100
50
% Cell Viability
B
BP — + + + + — + + + +
zVAD — — + + + — — + + +
Nec-1 — — — + — — — — + —
GSK — — — — + — — — — +

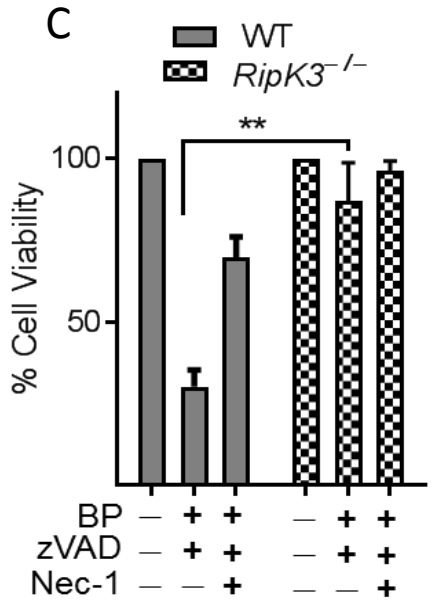
Figure 27. BP induced necroptosis is not impacted by prolonged differentiation. Monocytes and BMDMs (at day 5 and day 12 of growth) from WT (C57BL/6) mice were treated with BP (10µM), zVAD (50uM), GSK 872 (5µM) and Nec-1 (10µM) as indicated. After 24 hours incubation, the cell viability was measured by MTT assay. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

4.3.2. BP induced necroptosis is dependent on TNF-R1, IFNAR1 and RipK3 but not on the kinase region of RipK1

We wished to investigate the mechanism of BP induced necroptosis further and evaluated the role of TNF-R, IFNAR1, RipK1 and RipK3 signaling. Since, TNF-R1, RipK1 and RipK3 are indispensable for BP induced cell death, we evaluated the role of these signaling pathways in BP induced necroptosis following BP+zVAD treatment. Macrophages were generated form WT, TNF-R1-/-, TNF-R2-/-, TNF-R1/2-/-, RipK3-/- and $RipK1^{K45A}$ mice. D5 macrophages were treated with BP+zVAD with and without GSK or Nec-1 and the cell viability was measured at 24 hours by MTT assay. As expected, BP+zVAD treatment induced cell death in WT macrophages (Fig. 28A). TNF-R1-/- and TNF-R1/2-/- macrophages were partially but significantly resistant to BP+zVAD induced cell death while TNF-R2-/- macrophages were highly susceptible to cell death (Fig. 28A). IFNAR1 signaling has been reported to be indispensable for necroptosis induced by various stimulators (McComb et al., 2014). Our results indicated that macrophages that are deficient in IFNAR1 showed significant resistance to cell death induced by BP+zVAD as compared to the WT macrophages (Fig. 28B). While RipK3 deficient macrophages showed significant resistance to BP+zVAD induced cell death (Fig. 28C), $RipK1^{K45A}$ macrophages remained susceptible to BP+zVAD induced cell death although a slight resistance was observed (Fig. 28D). Taken together these results indicate that, like BP induced ripoptosis (ripoptosome-induced cell death), BP induced necroptosis is dependent on TNF-R1, but not TNF-R2, signaling. Interestingly, BP induced necroptosis is dependent on both TNF-R1 and IFNAR1 signaling. Further, BP induced necroptosis is dependent on RipK3, but not on RipK1. RipK1 kinase activity, which is indispensable for BP induced ripoptosis, doesn't seem to play a role in BP induced necroptosis


A
****
**
***
%Cell viability
100
50
WT
Tnfr1−/−
Tnfr2−/−
Tnfr1/2−/−
BP – + + – + + – + + – + +
zVAD – + + – + + – + + – + +
GSK – – + – – + – – + – – +


B
WT
Ifnar1−/−
***
% Cell Viability
100
50
BP – + + – + +
zVAD – + + – + +
Nec-1 – – + – – +


C
WT
RipK3−/−
**
% Cell Viability
100
50
BP – + + – + +
zVAD – + + – + +
Nec-1 – – + – – +

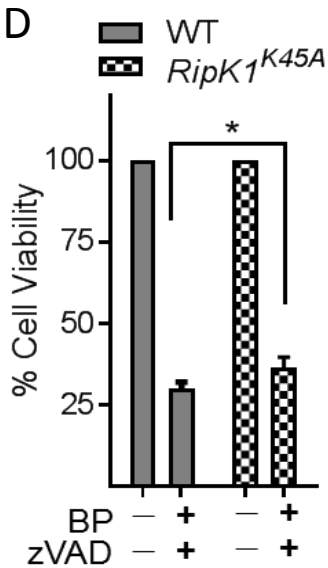
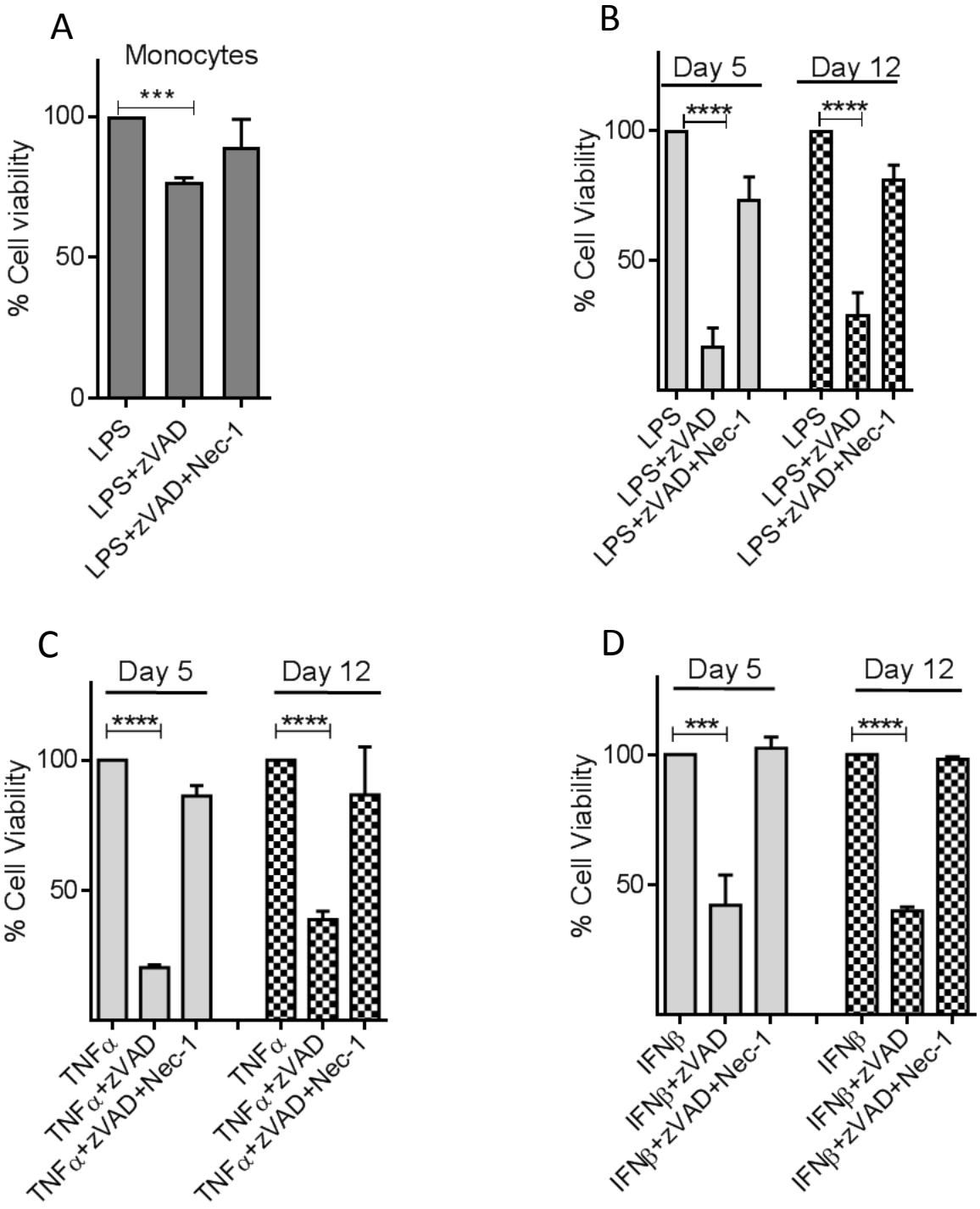

D
WT
RipK1K45A
*
% Cell Viability
100
75
50
25
BP – + – +
zVAD – + – +

Figure 28. Cell death induced by BP+zVAD is rescued by the deficiency of TNF-R1 and IFNAR1. Day 5 BMDMs from WT (C57BL/6), TNF-R1-/-, TNF-R2-/- TNF-R1/2-/-, IFNAR1-/-, RipK3-/- and RipK1^{K45A} mice were treated with (BP) (10μM), zVAD (50μM), GSK 872 (5μM) and Nec-1 (10μM) as indicated. After 24 hours incubation, the cell viability was measured by MTT assay. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

4.3.3. Macrophage differentiation does not impact necroptosis induced by TLR stimulation and cytokine signaling

Having evaluated the impact of macrophage differentiation on SM induced necroptosis, we were interested in determining the impact of macrophage differentiation on necroptosis induced by TLR4 stimulation and cytokine signaling. LPS, TNF-α and IFN-β in the presence of the pan-caspase inhibitor zVAD, has been previously reported to induce necroptosis in macrophages (McComb et al., 2014). We also evaluated the impact of LPS+zVAD on purified monocytes from bone marrow. Treatment of monocytes with LPS+zVAD induced a modest but significant cell death, which was rescued by Nec-1 (Fig. 29A). D5 and D12 macrophages were treated with LPS+zVAD, TNF-α+zVAD and IFN-β+zVAD with and without Nec-1. D5 macrophages were highly susceptible to cell death following LPS+zVAD (Fig. 29B), TNF-α+zVAD (Fig. 29C) and IFN-β+zVAD treatment (Fig. 29D). Addition of Nec-1 resulted in significant rescue of the cell death in macrophages (Fig. 29B, C, D). Interestingly D12 macrophages were also highly susceptible to LPS+zVAD, TNF-α+zVAD and IFN-β+zVAD treatment induced necroptosis (Fig. 29B, C, D). These results indicate that there is an increased susceptibility to LPS+zVAD induced necroptosis as the monocytes differentiate to macrophages, and the prolonged differentiation of macrophages doesn't result in resistance to necroptosis induced by LPS or cytokines TNF-α and IFN-β.

To investigate the mechanism of LPS+zVAD induced death further, we evaluated the role of TNF-R, IFNAR1 and RipK3 signaling in D5 macrophages. Our data indicated that like BP induced necroptosis, TLR4 mediated necroptosis in macrophages is dependent on


A
Monocytes
***
100
50
0
% Cell viability
LPS
LPS+zVAD
LPS+zVAD+Nec-1
B
Day 5
Day 12
****
****
100
50
% Cell Viability
LPS
LPS+zVAD
LPS+zVAD+Nec-1
LPS
LPS+zVAD
LPS+zVAD+Nec-1
C
Day 5
Day 12
****
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100
50
% Cell Viability
TNFα
TNFα+zVAD
TNFα+zVAD+Nec-1
TNFα
TNFα+zVAD
TNFα+zVAD+Nec-1
D
Day 5
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100
50
% Cell Viability
IFNβ
IFNβ+zVAD
IFNβ+zVAD+Nec-1
IFNβ
IFNβ+zVAD
IFNβ+zVAD+Nec-1

Figure 29. Macrophage differentiation doesn't alter their susceptibility to necroptosis induced by LPS, TNF-α and IFN-β. Monocytes and BMDMs (at day 5 and day 12 of growth) from WT (C57BL/6) mice were treated with LPS (1ng/ml), TNF-α (50ng/ml), IFN-β (1000U/ml), zVAD (50μM) and Nec-1 (10μM) as indicated. After 24 hours incubation, the cell viability was measured by MTT assay. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in three different mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

RipK3 and IFNAR1 signaling (Appendix-Fig. IVA, B), however, the cell death was not dependent on TNF-R signaling (Appendix-Fig. IVB).

4.3.4. Necroptotic signaling is not impacted by prolonged differentiation of macrophages

We also determined the impact of macrophage differentiation on necrosome signaling by western blot analysis. Necrosome signaling was evaluated by performing western blot analysis of cells stimulated with LPS+zVAD. We collected cell lysates from D5 and D12 WT BMDMs following treatment with LPS+zVAD for varying time intervals. Following LPS+zVAD treatment, the degradation of cIAPs was detected after 3 hours of treatment in both D5 and D12 cells (Fig. 30A). The densitometric analysis indicated that while the expression levels of cIAPs was higher in day 12 macrophages, the degradation was similar in macrophages at both stages of differentiation (Fig. 30B). The phosphorylation of RipK1 was detected as early as 1 hour post stimulation in D5 macrophages and interestingly there was sustained activation of RipK1 in D12 macrophages as well (Fig. 30A). Similarly, the phosphorylation of RipK3 was detected at 3 hour of stimulation with LPS+zVAD in D5 macrophages and similar phosphorylation was detected in D12 macrophages (Fig. 30A). The densitometric analysis of phosphorylated RipK1 to total RipK1 and phosphorylated RipK3 to total RipK3 clearly revealed that there was similar phosphorylation detected in RipK1 and RipK3 in D5 and D12 macrophages (Fig. 30C and D). These results indicate that RipK1 and RipK3 which are the key proteins of necroptosis signaling are not impacted by prolonged differentiation of macrophages, and this correlates with their susceptibility to LPS+zVAD induced necroptosis.

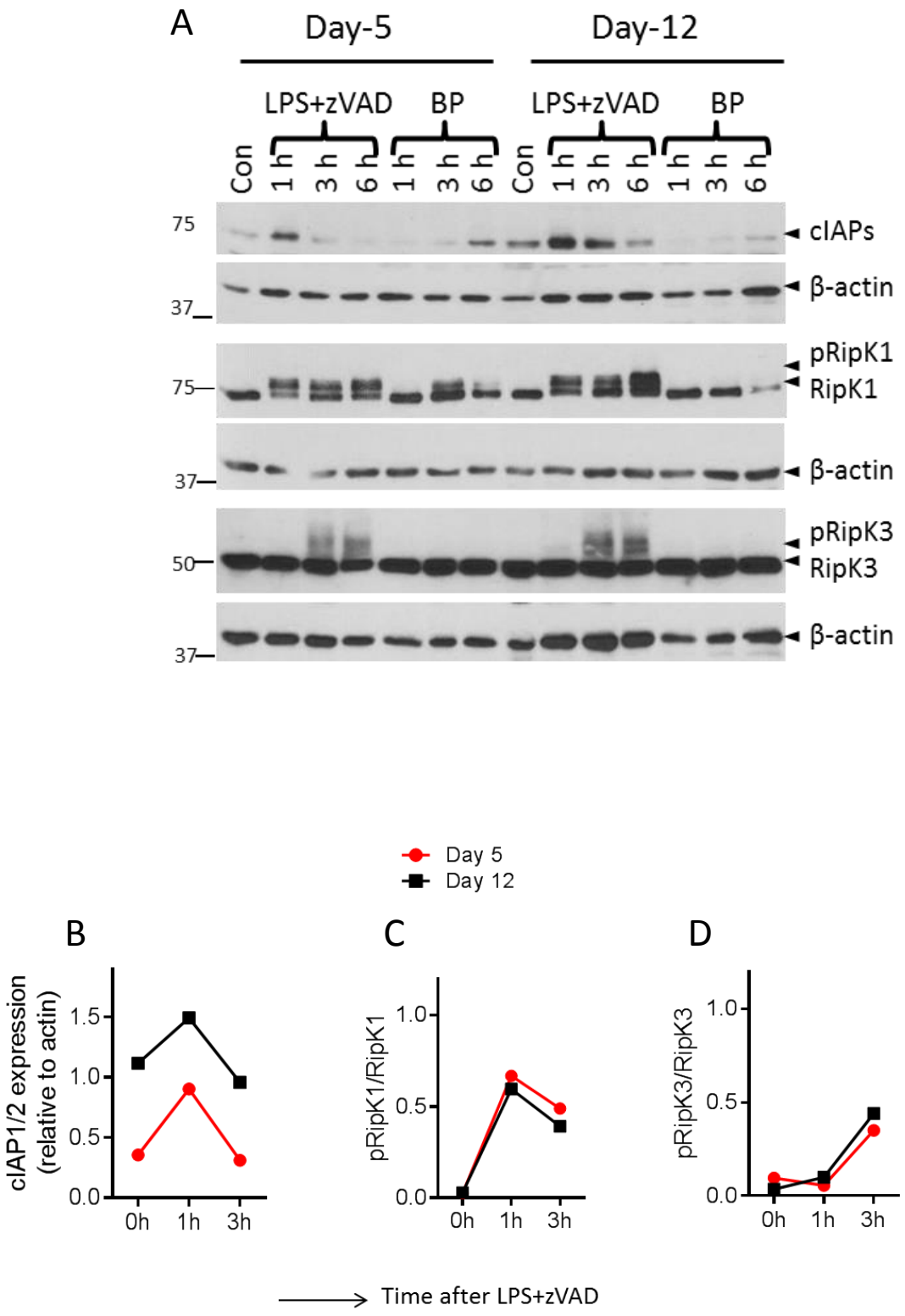
A
Day-5
Day-12
LPS+zVAD
BP
LPS+zVAD
BP
Con
1 h
3 h
6 h
1 h
3 h
6 h
Con
1 h
3 h
6 h
1 h
3 h
6 h
75
cIAPs
β-actin
37
75
pRipK1
RipK1
β-actin
37
50
pRipK3
RipK3
β-actin
37
Day 5
Day 12
B
cIAP1/2 expression (relative to actin)
1.5
1.0
0.5
0.0
0h
1h
3h
C
pRipK1/RipK1
1.0
0.5
0.0
0h
1h
3h
D
pRipK3/RipK3
1.0
0.5
0.0
0h
1h
3h
Time after LPS+zVAD

Figure 30. RipK1 and RipK3 phosphorylation is not impacted by prolonged differentiation of macrophages. Lysates were collected from LPS (1ng/ml) +zVAD (50μM) treated WT macrophages over the course of 6 hours (as indicated), on day 5 and day 12 of differentiation to measure cIAPs, RipK1, RipK3 and β-actin by western blot (A). The buffer used for lysing was SDS buffer. Densitometric ratios of cIAPs normalised to actin (B), phosphorylated RipK1 to unphosphorylated RipK1 (pRipK1/RipK1) (C) and pRipK3/RipK3 (D) following LPS and LPS+BP treatment were calculated. The densitometric analysis is shown only for the first three-hour post treatment for each protein. All western blot experiments were done at least three times in three different mice.

5.0. DISCUSSION

Cell death and survival in monocyte/macrophage lineage play a fundamental role in the initiation and resolution of inflammation in the host. Cell death in macrophages is an effective means of regulating immune response by many intracellular bacteria, however, the type and the context of cell death determines the overall host pathogen interactions (Sanz et al., 2014). While apoptosis was previously reported to be a central mechanism of cell death, various alternative mechanisms of cell death have been reported in the last decade. Pyroptosis in macrophages has been reported in case of many bacterial infections, and this was previously confused with apoptosis owing to some similarity in the pathways involved (Lai et al., 2015). Similarly, necroptosis in macrophages has been reported during infection with *Salmonella enterica* Typhimurium (Robinson et al., 2012). Stimulation of cells with IFN-1 (Robinson et al., 2012), SMAC mimetics (McComb et al., 2012), or TLR3/4 activators has also been shown to induce necroptosis (He et al., 2011).

Ripoptosome signaling complex is another cell death inducing complex that was discovered recently which involves proteins that are involved in both apoptosis and necroptosis pathways. Genotoxic stress, SMAC mimetic, etoposide treatment or TLR3 activation results in ripoptosome complex formation and cell death (Feoktistova et al., 2011; Tenev et al., 2011). It is very well established that RipK1 is the indispensable component of this complex (Feoktistova et al., 2011; Tenev et al., 2011). In my own experiments I have observed there is complete resistance to ripoptosome-induced cell death (in response to SMAC mimetic, BP) in the absence of RipK1 kinase activity (Fig. 5B, 7B). Although ripoptosome has not been reported to be induced in response to bacterial infection in macrophages, it was recently reported that RipK1 was a key upstream mediator in inducing capsase-8 dependent apoptosis or RipK3 dependent necroptosis in macrophages in response

to *Yersinia* infection (Weng et al., 2014). Nec-1 has been shown to have a protective role against many diseases such as ischemic brain injury (Degterev et al., 2005), neonatal hypoxia-ischemia (Chavez-Valdez et al., 2012; Northington et al., 2011), myocardial (Smith et al., 2007) and renal ischemia (Linkermann et al., 2012). Since RipK1 is the central component of the ripoptosome complex, there have been speculations that RipK1 might be playing a critical role in the pathogenesis of these diseases through ripoptosome, rather than necrosome signaling (Blander, 2014). Most of the studies addressing the mechanism of ripoptosome signaling have been done in cancer cell lines. The elevated levels of cIAPs and the consequent resistance to cell death in cancer cells make them perfect models for studying ripoptosome signaling (Almagro and Vucic, 2012).

It is equally important to decipher the mechanism of this complex signaling in the immune cells. Monocytes and macrophages are both important cell types in the innate immune response. Monocytes differentiate to macrophages, but the relative time that a macrophages takes to differentiate is not fully clear. In our research, we investigated the susceptibility of monocytes and macrophages (at different stages of differentiation) to necrosome and ripoptosome signaling. Interestingly our results indicated that monocytes and macrophages respond differentially to these two cell death inducing complexes. We observed that prolonged differentiation of macrophages results in acquisition of resistance to ripoptosome signaling-induced cell death that is induced through increased expression of the inhibitory proteins XIAP and $cFLIP_L$.

5.1. Differentiation of macrophages

Most of the research on macrophages is done with *in vitro* derived macrophages from bone marrow precursors, primarily due to increased cell yield of differentiated macrophages and the ease of purification (Ohradanova-repic et al., 2016). We used four cell surface markers (CD11b, F4/80, Ly6C and Ly6G) that are used commonly in specifically discriminating macrophages from other immune cells (Murray and Wynn, 2011). We observed that as monocytes differentiated into macrophages *in vitro*, there was up-regulation of F4/80 expression, which is a classic marker for murine macrophages (Murray and Wynn, 2011), while the expression of Ly6C, that is highly expressed by the monocytes (Ramachandran et al., 2012), was diminished (Fig. 4A). In an *in vitro* study, the expression of F4/80 was shown to increase in macrophages at day 7 post differentiation from bone marrow precursors, and this expression of F4/80 did not change subsequently after continued differentiation (Leenen et al., 1994). This is in agreement with our finding as we did not observe any change in F4/80 expression after day 5 of differentiation (Fig. 4A). CD11b has been shown to be another important differentiation marker in macrophages (Chamberlain et al., 2015), and we also observed a relative increase in the expression of CD11b during differentiation of macrophages (Fig. 4A). Interestingly, we observed that unlike F4/80, the expression of CD11b continued to increase after day 5 of differentiation (Fig 4B). It was previously reported that the expression of CD11b remained constant during the differentiation of macrophages from day 7 to day 21 in murine BMDMs (Chamberlain et al., 2015). In contrast, in another study, CD11b expression was reported to increase with differentiation of macrophages *in vitro* (Walker et al., 1985). Recently, CD11b expression was reported to be reduced in macrophages of cystic fibrosis patients which were also less phagocytic, indicating that the expression of CD11b may be an important marker for

effective macrophage function (Jeune et al., 2013). Macrophages and monocytes lacked expression of Ly6G thus ruling out the presence of any neutrophils in the cell population (Fig. 4A).

The protocols used for differentiation of macrophages vary as some investigators use L929 supernatant to differentiate macrophages whereas others use M-CSF, and the impact of this difference may also influence the speed of differentiation of macrophages. In most of the studies, BMDMs of mouse are generated after 5-7 days of differentiation in response to L929 supernatant or M-CSF. Some studies are also done on macrophages grown until day 10 (Warren and Vogel, 1985) or day 21 (Chamberlain et al., 2015). Similarly, human monocyte derived macrophages are also generated by growing peripheral blood mononuclear cells (PBMCs) for 7 days (Chomarat et al., 2000; Ohradanova-repic et al., 2016). In a study done by Vogt and Nathan (2011), they used human monocyte-derived macrophages at 14 days of differentiation to study the impact of macrophages on *Mycobacteria.* Interestingly, they found that macrophages with extended differentiation in response to GM-CSF developed the ability to reduce the replication of these bacteria *in vitro* compared to the day 7 macrophages (Vogt and Nathan, 2011). It is thus interesting to see the difference in macrophage functions relative to the time of its *in vitro* differentiation. There is no consensus regarding the time required for complete differentiation of macrophages, and how this impacts their function.

5.2. Only ripoptosome is inhibited

Very few studies on ripoptosome signaling have been done in immune cells such as macrophages. We used two SMAC mimetics (SMs), BP and BV6 to induce ripoptosome signaling in D5 and D12 macrophages. Birinapant (BP) has been shown to be very effective in inducing cell death of cancer cell lines (Benetatos et al., 2014) and BMDMs (Shutinoski et

al., 2016). In our experiments, we observed that D5 macrophages were highly susceptible to ripoptosome-induced cell death in response to both the SMs, while D12 macrophages were resistant to the cell death (Fig. 5B). In a study done by Muller-Sienerth et al. (2011) on human monocytes and macrophages, they observed that BV6 induced cell death in monocytes but not in macrophages, which doesn't agree with our finding with murine macrophages. In a study done on 50 cancer cell lines, 22% of cells were susceptible to SM induced cell death while 78% of the cell lines were resistant to cell death. The resistance to cell death correlated with lesser interaction of the SM with cIAPs (as shown by the pull down experiment of the interacting proteins using biotinylated form of SM) and also reduced autocrine production of TNF-α (Petersen et al., 2007). In another study Mass et al. (2013) demonstrated that the resistance of chronic lymphocytic leukemia (CLL) cells to cell death in response to two different SMs is due to an absence of ripoptosome formation even though there was effective degradation of cIAPs. This is in agreement with our finding which shows that effective degradation of cIAPs in D12 macrophages doesn't render them susceptible to cell death (Fig. 9A).

In addition to cell death by ripoptosis (ripoptosome-induced cell death), we also investigated whether cell death by the alternative pathway of necroptosis is impacted following stimulation with LPS+zVAD, TNF-α+zVAD, IFN-β+zVAD and BP+zVAD. Consistent with previous findings in macrophages (McComb et al., 2014), all the necroptotic stimulators used in our experiments were able to induce necroptosis in D5 macrophages which was rescuable by Nec-1 or GSK (Fig. 27B, 29A, C, D). Resistance to necroptosis has been reported in many cases. In a study done by He et al. (2009), they showed inhibition of necroptosis *in vitro* and *in vivo* in the absence of RipK3. Similarly, CLL cells have been

reported to be resistant to necroptotic signaling in response to TNF-α+zVAD treatment due to the downregulation of RipK3 and CYLD (Liu et al., 2012). Interestingly, in response to all the stimulators that we used, macrophages that were differentiated for extended periods were equally susceptible to necroptosis and this correlated with similar phosphorylation of the key proteins, RipK1 and RipK3 (Fig. 30A, C, D). Importantly we observed that the ripoptosome-induced death that was inhibited in D12 macrophages didn't switch to necroptosis. We speculate that this may be due to the high levels of $cFLIP_L$ that we observed in our results (Fig. 9A), as $cFLIP_L$ is known to regulate apoptosis and necroptosis both by the cleavage of RipK1 in the ripoptosome complex (Feoktistova et al., 2011). Taken together, our finding raise a key question as to why macrophages develop resistance to ripoptosis with extended differentiation without any corresponding impact on susceptibility to necroptosis.

5.3. The role of TNF-α in ripoptosome signaling

The autocrine secretion of TNF-α leading to TNF-R1 signaling has been reported to be indispensable in SM induced cell death in most of the studies done on cancer cell lines (Petersen et al., 2007; Probst et al., 2010; Varfolomeev et al., 2007) and in macrophages (McComb et al., 2012). In contrast, a study done on HaCaT cells using SM along with Poly (I:C), an antagonist of TLR3, reported that the ripoptosome formed is recruited to TLR3 and the consecutive cell death is not dependent on TNF-R1 signaling (Feoktistova et al., 2011). TNF-α independent ripoptosome-induced cell death was also observed by Tenev et al. (2011) in response to etoposide treatment. In our studies we found that while SM induced cell death was completely inhibited in TNF-α-/- and TNF-R1-/- macrophages (Fig. 6A, B), cell death was induced even in the absence of these signaling mechanisms when TLR4

signaling was engaged in conjunction with SMAC treatment (Fig. 23A, B). Thus, the mechanism of cell death induced by SMAC treatment can vary depending on the stimuli. Peterson et al. (2007) have reported the resistance to SM induced cell death in cancer cells due to ineffective TNF-α production. Thus in an attempt to see if lower TNF-α expression was the reason for reduced cell death in D12 macrophages, we measured TNF-α production in macrophages at both the stages of differentiation. Surprisingly, the secretion of TNF-α was similar in D5 and D12 macrophages (Fig. 6C). This is in agreement with a study where the SM treated chronic lymphocytic leukemia cells showed effective TNF-α production but were resistant to cell death (Maas et al., 2013).

It is possible that the resistance of D12 macrophages to cell death was not due to the reduced TNF-α expression but due to the reduced TNF-R1 signaling or downstream of TNF-α/TNF-R1 signaling. Consistent with this, we observed that RipK1 and p38 MAPK undergo reduced phosphorylation following SMAC treatment in D12 macrophages. Furthermore, TNF-α-signaling was required for phosphorylation of RipK1 (Fig. 21A, B), and MK2/ p38 MAPK (Fig. 21A, C). These results indicate that TNF-α promotes p38 MAPK and MK2 phosphorylation, which in turn phosphorylates RipK1 (Fig. 19C, E). Again this finding raises the question whether downstream signaling following TNF-α expression could be the possible reason for reduced RipK1, p38 and MK2 phosphorylation that we observe in D12 macrophages. The phosphorylation of RipK1 and its role in SM induced cell death will be discussed in detail in the next section.

Interestingly, in the absence of MK2, high TNF-α production was observed that correlated with enhanced cell death in D12 macrophages (Fig. 19F). Similar results were reported recently by Lalaoui et al. (2016) emphasizing the role of p38 MAPK and MK2 in

regulating TNF-α production in response to SM treatment. This is in sharp contrast to the role of these kinases in TNF-α production during TLR signaling where it has been reported that p38 MAPK and MK2 promote maintenance of TNF-α (Dumitru et al., 2000; Kotlyarov et al., 1999). Thus, the impact of p38 MAPK and MK2 on TNF-α expression is dependent on cell stimulation, and the reason for this is not clear. Taken together our results indicate that TNF-α promotes the phosphorylation of MK2 following SMAC treatment, and MK2 negatively regulates TNF-α. Thus it is expected that D12 macrophages which express lower pMK2, the inhibitor of TNF-α, should express higher levels of TNF-α. However, this is not the case, as the production of TNF-α is similar in D5 and D12 macrophages. Since multiple mechanisms promote TNF-α production, it seems likely that a subset of mechanisms that promote TNF-α production is attenuated in D12 macrophages. This could be due to decreased canonical NF-kB activation in D12 macrophages observed in our experiments (Fig. 10A, C).

5.4. The role of RipK1 versus RipK3 in ripoptosome signaling

Many studies have shown the indispensable role of RipK1 in ripoptosome-induced cell death (Christofferson et al., 2014), which distinguishes this form of cell death to the extrinsic pathway of apoptosis. Necrostatin-1 (Nec-1) was identified as the inhibitor of RipK1 kinase function (Degterev et al., 2008) and its role in the inhibition of ripoptosome-induced cell death has been previously described (Ofengeim and Yuan, 2013). In agreement to the previous findings, our experiments showed that the SMAC mimetic (BP and BV6), induced cell death is dependent on RipK1 kinase activity and is rescued by Nec-1 (Fig. 5B, Appendix-IA). The cell death with BP was also completely inhibited in RipK1 kinase

inactive (RipK1^{K45A}) macrophages (Fig. 7B). Although it is known that the kinase function of RipK1 is required for ripoptosome-induced cell death, the specific mechanism of RipK1 kinase in the formation of ripoptosome is not very clear. In a study done by Degterev et al. (2008), they identified that the various auto-phosphorylation sites of RipK1 are located in the kinase region of RipK1, indicating that the kinase activity of RipK1 might be leading to its auto-phosphorylation. One of the interesting finding in our results is that there is RipK1 phosphorylation post BP treatment in D5 macrophages while D12 macrophages express very low RipK1 phosphorylation (Fig. 8A, C). In a study done previously in our lab it has been shown that RipK1 phosphorylation following BP treatment is completely inhibited in RipK1^{K45A} macrophages (Shutinoski et al., 2016). These findings indicate that RipK1 is auto-phosphorylated following treatment with SMAC mimetic. As discussed earlier in my previous section, poor expression of p38 MAPK and MK2 in D12 macrophages also explains why phosphorylation of RipK1 is reduced in D12 macrophages (Fig. 10A). Phosphorylation of RipK1 mediated by p38 MAPK or MK2 has not been reported till date. Our results indicate that the phosphorylation of RipK1 post BP treatment, is either a combined effect of auto-phosphorylation and phosphorylation by p38 MAPK/MK2, or mediated by the cross talk between the two. Thus we speculate that inhibition in RipK1 phosphorylation in D12 macrophages may inhibit the RipK1 kinase function thus restricting the formation of ripoptosome complex.

Since Nec-1 blocks RipK1 kinase region and prevents RipK1-RipK3 interaction (Christofferson et al., 2014), we investigated the role of RipK3 in BP induced cell death. Interestingly, our results indicated that the cell death is dependent on RipK3 scaffold but not its kinase function (Fig. 7A, C, and D). Similar results were reported by Dondelinger et al. (2013) in TNF+SM treated MEF cells. They showed that in case of RipK3 deficiency,

caspase 8 and caspase 3 activation was limited, indicating the role of RipK3 in caspase 8 mediated apoptosis in SM treated cells. In our own co-immunoprecipitation experiments using antibody against caspase 8, we found that the interaction of caspase 8 and RipK3 was more pronounced in D5 macrophages while it was diminished in D12 macrophages (Fig. 12A), indicating the role of RipK3 in BP induced cell death. Together, our data indicates a novel mechanism of phosphorylation of RipK1 by p38 MAPK/MK2 that is important in ripoptosome signaling and how macrophages with extended differentiation are subjected to reduced phosphorylation leading to resistance to cell death.

5.5.The role of caspases in ripoptosome signaling:

It is well established that caspase 8 is responsible for inducing cell death in the ripoptosome complex (Blander, 2014). When we measured caspase 8 activation by western blotting, we found that BP induced the activation of caspase 8 in D5 macrophages and this activation was significantly reduced in D12 macrophages (Fig. 11A). Similar results were seen with caspase 8 luminescence bioassay as well (Fig. 11B). We were unable to detect activated form of caspase 3 and caspase 9 by western blot (data not shown), however we measured caspase 3/7 and caspase 9 activity by luminescence assay. Along with caspase 8, there was enhanced activation of caspase 9 and caspase 3/7 in response to BP treatment of D5 macrophages (Fig. 13B, C). In a study done by Peterson et al. (2007) in human lung cancer cell line, they reported the activation of caspase 8 and caspase 9 at 4h post-SM treatment, however only the siRNA knockdown of caspase 8 rescued the cell death but the knockdown of caspase 9 had no impact, indicating the role of caspase 8 as the initiator caspase in this case. Similar results were reported in another study (Gaither et al., 2007). Interestingly, our results indicate that the enhanced activities of caspase 8, 9 and 3/7 were

completely abrogated in RipK1^{K45A} macrophages (Fig. 13A, B, and C). These data indicate that the kinase region of RipK1 is the upstream activator of all of these caspases, and that caspase 8 activation also activates caspase 9 during ripoptosome signaling. Although, RipK1 interacts with caspase 8 to form the ripoptosome complex, we couldn't detect RipK1 associated with caspase 8 in co-immunoprecipitation experiment done on caspase 8 (Fig. 12A). In contrast to our results, enhanced interaction of RipK1 and caspase 8 was seen in the immunoprecipitation experiments done in other studies (Feoktistova et al., 2011; Tenev et al., 2011). In both the studies they used zVAD to stabilize the ripoptosome complex. Since we didn't use zVAD in our experiments, the absence of RipK1 in our results could be due to the instability of the complex. It could be also due to the transient interaction of RipK1 with caspase 8 and its rapid cleavage by activated caspase 8.

XIAP and cFLIP$_L$ have been reported to regulate ripoptosome formation as the enhanced formation of ripoptosome was observed after the siRNA knockdown of XIAP and cFLIP$_L$ in cancer cells after SM treatment (Feoktistova et al., 2011; Tenev et al., 2011). In our experiments, we found elevated levels of XIAP and cFLIP$_L$ in D12 macrophages that correlated to cell death resistance (Fig. 9A). XIAP has been reported to be elevated in many cancer cell lines (Hunter et al., 2007). In one of the studies done in chronic lymphocytic leukemia (CLL) cells, there was restoration of apoptotic cell death with XIAP inhibitor (Kater et al., 2005) indicating the role of XIAP in cell death resistance. When we used XIAP-/- macrophages, the cell death resistance in D12 macrophages was completely inhibited and cell death was restored (Fig. 14A). XIAP is known to inhibit caspase 3, 7 and 9 (Darding and Meier, 2012) and inhibit apoptosis. Thus we investigated the impact of XIAP deficiency on the caspase activity in D12 macrophages. Interestingly, caspase 8, caspase 9 and caspase 3/7 activities were highly enhanced in XIAP-/- macrophages (Fig. 14B, C, D). It was interesting

to see that XIAP was regulating caspase 8 activation along with caspase 3/7 and caspase 9 in D12 macrophages. It is hard to infer, if XIAP is directly regulating caspase 8 or indirectly regulating it through caspase 3, since caspase 3 has been shown to have a feedback mechanism to activate caspase 8 (Ferreira et al., 2012). Our experiments also revealed increased caspase 8-cFLIP$_L$ interaction in D12 macrophages in the co-immunoprecipitation experiment (Fig. 12A). cFLIP$_L$ is the direct inhibitor of caspase 8 in the ripoptosome complex (Feoktistova et al., 2011). Thus, the interaction of cFLIP$_L$ with caspase 8 indicates its role in inactivating caspase 8 in D12 macrophages. It is not clear which of these inhibitory molecules (cFLIP$_L$ or XIAP) is the dominant mechanism that is responsible for the cell death resistance in D12 macrophages, and it is quite possible that the potent resistance to cell death is likely due to synergistic impact of these two inhibitors. The expression of XIAP and cFLIP$_L$ is known to be promoted by NF-kB activation (Kreuz et al., 2001b; Stehlik et al., 1998). However, in our experiments, activation of the canonical NF-kB pathway was not enhanced in D12 macrophages. In contrast, the non-canonical NF-kB pathway was enhanced in D12 macrophages, which could possibly be responsible for increased XIAP and cFLIP$_L$ expression. Interestingly, we observed that p38 and MK2 MAPK pathways appeared to regulate caspase 8 activation and cell death in D12 macrophages (Fig. 15A, 16A, 19B). Recently, p38 MAPK signaling was shown to regulate caspase 8 activation in acute myeloid leukemia (AML) cells in vitro (Lalaoui et al., 2016).

5.6. The role of p38 MAPK and MK2 in caspase activation and cell death

As mentioned previously, inhibition of p38 MAPK or the absence of MK2 resulted in restoration of ripoptosome-induced cell death in D12 macrophages with enhanced TNF-α

production (Fig. 19F) and caspase 8 activation which is in agreement with the finding of Lalaoui et al. (2016). The role of p38 MAPK seems to be highly ambiguous in the context of caspase 8 activation. Activated p38 MAPK was reported to induce caspase 8 activation and cell death in response to sphingosine in Jurkat T cells (Yoon et al., 2009). In contrast, another study reported inhibition of caspase 8 by p38 MAPK in human neutrophils in response to FAS ligation (Alvarado-kristensson et al., 2004).

According to our results, it seems that p38 MAPK and MK2 have dual function, one is to phosphorylate RipK1 which induces ripoptosome formation and cell death following caspase 8 activation. The other role is to limit caspase 8 activation by as yet unknown mechanism, as inhibition of p38 MAPK/MK2 enhances caspase 8 activation in D5 and D12 macrophages (Fig. 16A, 19B). The lower levels of p38 MAPK and MK2 correlate with reduced phosphorylation of RipK1 in D12 macrophages (Fig. 8A, 10A) that may promote reduction of ripoptosome formation in these cells. On the other hand, due to the reduction in p38 MAPK and MK2 and knowing that these kinases regulate caspase 8, we expect more caspase 8 activity in D12 macrophages. Since the caspase 8 activation was not high but was significantly reduced in D12 macrophages (Fig. 11A), we assume that the higher levels of $cFLIP_L$ and XIAP might dominate and regulate caspase 8 activation in this context. However, despite the reduced levels of p38 MAPK or MK2 in D12 macrophages, when these kinases are further inhibited, there is enhanced cell death (Fig. 15A, 19A) which correlates with increased caspase 8 activation (Fig. 16A, 19B) even in the absence of phosphorylated RipK1 (Fig. 8A) and in the presence of high XIAP and $cFLIP_L$ levels (Fig. 9A). It is quite likely that p38 MAPK or MK2 regulate ripoptosome signaling through an alternative mechanism that is not clear currently. We observed increased JNK and ERK signaling in D12 macrophages which was further enhanced by inhibition of p38 MAPK (Fig. 18A).

These results suggest that p38 MAPK regulates ERK and JNK. However, inhibition of ERK or JNK did not restore cell death in D12 macrophages (Fig. 18F, G) suggesting that modulation of JNK or ERK is not responsible for the resistance of D12 macrophages. Seo et al. (2007) have reported sulindac-induced apoptosis in human multiple myeloma (MM) cells and the cell death correlated with p38 MAPK activation and downregulation of XIAP. We were interested to see if enhanced caspase activation with MK2 inhibition is due to reduction in endogenous caspase 8 inhibitors, XIAP and $cFLIP_L$. Our results indicated that there was no reduction in the levels of XIAP and $cFLIP_L$ with p38 MAPK or MK2 inhibition (Fig. 17A, 19C). Interestingly, our results indicated that XIAP regulates MK2, as enhanced MK2 phosphorylation was observed in D12 XIAP-/- macrophages (Fig. 20A). We speculate that the elevated levels of XIAP could be the reason of reduced MK2 phosphorylation in D12 macrophages. It has also been previously reported that XIAP and cIAP1 regulate MEKK2/3-MEK5-ERK5 signaling through ubiquitination (Takeda et al., 2014). These data indicate that XIAP have a significant role in regulating the MAPKs signaling and impacting cell death.

5.7. Concurrent TLR4 activation with BP treatment doesn't rescue the ripoptosome-induced cell death

TLR4 activation is known to induce cell survival in macrophages (Lombardo et al., 2007). We wanted to see if concurrent activation of TLR4 along with BP treatment will rescue the cell death induced by ripoptosome signaling. In a very recent study, it was demonstrated that BP treatment improved survival of mice from LPS induced liver injury by attenuating the pro-inflammatory cytokines production by kupffer cells (Liu et al., 2017). In contrast, in another study administration of SM, LCL-161 along with LPS increased the production of inflammatory cytokines leading to endotoxic shock in mice (West et al., 2016).

Our results showed that LPS+BP treatment didn't rescue cell death, but induced cell death in monocytes and macrophages that was only partially dependent on RipK1 kinase activity (Fig. 22A, B) which is in contrast to BP induced cell death. Further, the cell death was also only partially dependent of TNF-α/TNF-R1 signaling (Fig. 23A, B). However, similar to BP induced cell death, RipK3 scaffold was required for this form of cell death (Fig. 23D). In a study done by Lawlor et al. (2015), IAPs depletion and LPS stimulation induced RipK3 scaffold mediated caspase 8 activation and apoptosis in BMDMs. They demonstrated that this form of cell death is highly suppressed by XIAP and thus can take place only in case of complete deletion of XIAP. Interestingly, we found that D12 macrophages developed resistance to LPS+BP induced cell death as well (Fig. 22B) and the resistance correlated with high XIAP levels (Fig. 25A). In agreement to their study, we found that in case of XIAP deficiency, the cell death was restored in D12 macrophages (Fig. 25E). Along with XIAP, the $cFLIP_L$ level was also elevated in D12 macrophages (Fig. 25A). When we measured caspase activation, we observed reduced caspase 8, caspase 9 and caspase 3/7 activation in D12 macrophages treated with LPS+BP (Fig. 26B, C, D). This attenuation in caspase activation could be due to the combined impact of XIAP and $cFLIP_L$ as was observed in BP induced cell death.

Interestingly, phosphorylation of RipK1 was not abrogated in D12 macrophages treated with LPS+BP (Fig. 24A), indicating that the impairment in RipK1 phosphorylation observed in D12 macrophages in the context of ripoptosome signaling is restored with the addition of LPS although there is no cell death mediated by consistent RipK1 phosphorylation. Taken together our results indicate that ripoptosome signaling along with TLR4 activation induces canonical apoptosis which is partially RipK1 dependent, however

the elevated levels of the anti-apoptotic proteins, XIAP and $cFLIP_{L,}$ in macrophages regulate cell death induced by concurrent stimulation with LPS+BP.

6.0. CONCLUSION

The work presented in this thesis reveals some novel insights into the mechanisms that regulate ripoptosome signaling during differentiation of monocytes to macrophages. We have revealed that prolonged differentiation of macrophages results in resistance to ripoptosome-induced cell death. There was no impact on susceptibility to cell death by the necrosome pathway. In accordance with previous work in other cell types, we show that TNF-α/TNF-R1 signaling is indispensable for ripoptosome signaling in macrophages. However, differentiated macrophages expressed high levels of TNF-α, suggesting that the resistance of differentiated macrophages to ripoptosome-induced cell death is not related to the expression of TNF-α. We show that the kinase activity of RipK1 and RipK3 scaffold promote ripoptosome signaling in macrophages. Our results indicate that the resistance of differentiated macrophages to cell death by the ripoptosome pathway correlates with reduced phosphorylation of RipK1, increased expression of the anti-apoptotic mediators XIAP and $cFLIP_L$, and reduction in caspase 8, 9 and 3/7 activation. Further experiments revealed that the phosphorylation of RipK1 was mediated by p38 MAPK/MK2, and the levels of p38 MAPK/MK2 were reduced in differentiated macrophages. There was increased interaction of $cFLIP_L$ with caspase 8 in differentiated macrophages, which provides a mechanistic explanation of their resistance to cell death by the ripoptosome pathway. Furthermore, deficiency of XIAP restored the caspase activation and ripoptosome-induced cell death in differentiated macrophages.

Our results also revealed the contradictory role of p38 MAPK/MK2 in caspase 8 activation and cell death in the context of ripoptosome signaling. On one hand, p38 MAPK/MK2 signaling appears to activate caspase 8 by phosphorylating RipK1, while on the other hand, p38 MAPK/MK2 signaling also inhibits caspase-8 activation and TNF-α expression. These kinases seem to regulate JNK and ERK activation although the role of JNK and ERK in cell death was not pronounced. Our study also showed that XIAP regulates MK2 activation. Thus, in addition to directly inhibiting caspases, XIAP also regulates caspase 8 activation indirectly by limiting MK2 activation and consequently limiting RipK1 phosphorylation. Our results also revealed that ripoptosome signaling along with TLR4 activation doesn't rescue the ripoptosome-induced cell death but rather induces apoptosis which is only partially dependent on RipK1 signaling. Differentiated macrophages were also resistant to cell death induced by this stimulation, which was mediated by increased expression of XIAP and $cFLIP_L$.

Overall, the results of this thesis provide some new mechanisms of regulation of ripoptosome signaling in macrophages and provides a different outlook on macrophage differentiation and their responses to different cell death inducing complexes. The SMAC mimetic induced cell death may be exploited to treat acute myeloid leukemia and other diseases associated with dysfunctional macrophages. The knowledge on activation and regulation of ripoptosome signaling may also help to understand the tumor biology and the immune responses and develop therapeutic approaches for virus infections as well.

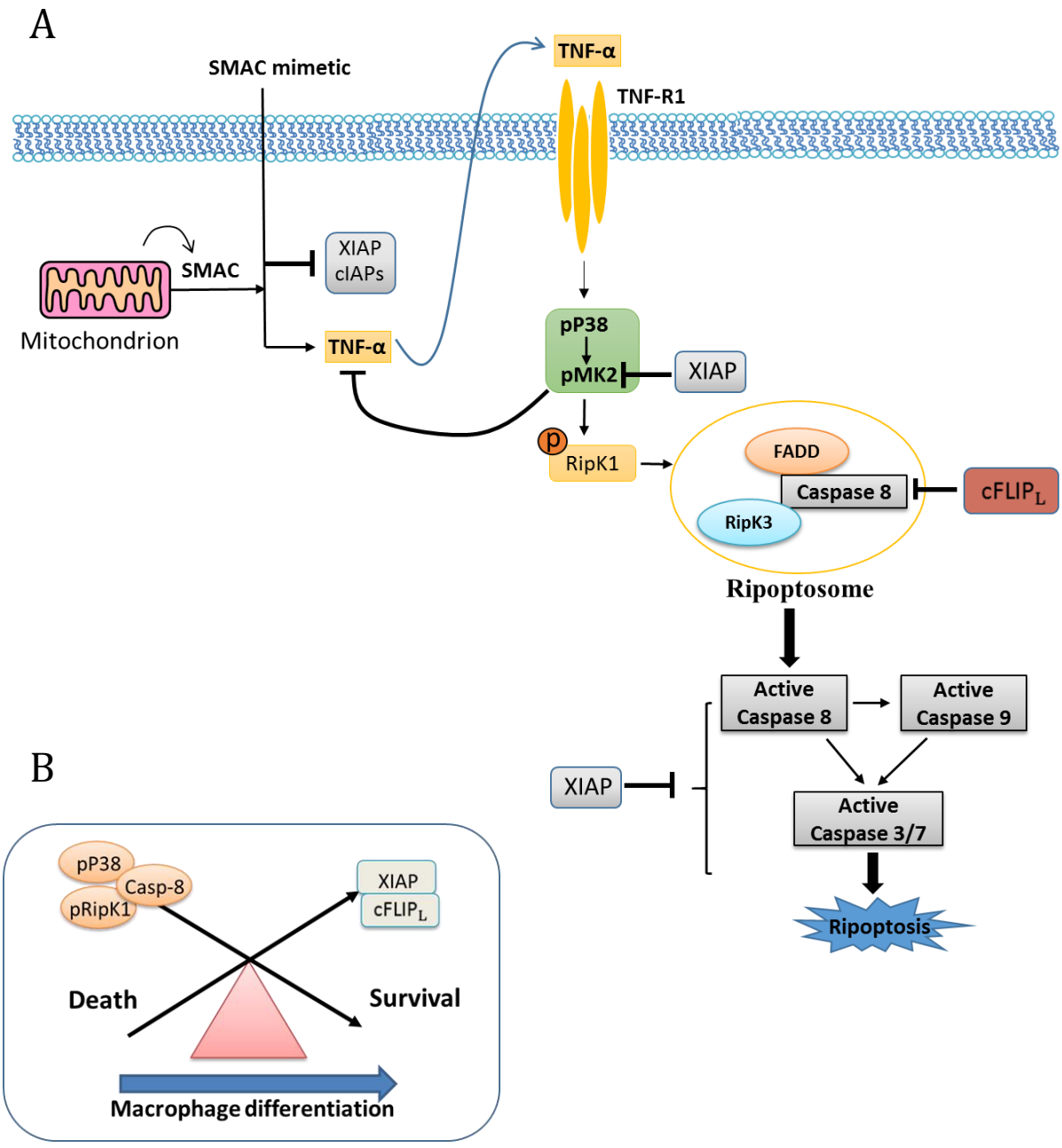
A
SMAC mimetic
TNF-α
TNF-R1
SMAC
XIAP
cIAPs
Mitochondrion
TNF-α
pP38
pMK2
XIAP
p
RipK1
FADD
Caspase 8
cFLIP$_L$
RipK3
Ripoptosome
Active Caspase 8
Active Caspase 9
XIAP
Active Caspase 3/7
Ripoptosis
B
pP38
Casp-8
pRipK1
XIAP
cFLIP$_L$
Death
Survival
Macrophage differentiation

Figure 31. Model of the regulation of ripoptosome signaling in BMDMs undergoing prolonged differentiation. SMAC protein or SMAC mimetic induces TNF-α production in macrophages that initiates the TNF-R1 signaling by autocrine fashion. TNF-R1 signaling mediates phosphorylation and activation of p38 MAPK and MK2 that further mediates RipK1 phosphorylation. The activated RipK1 forms the ripoptosome complex. Activation of caspase 8 in the ripoptosome complex further activates caspase 9 and caspase 3/7 and induces ripoptosome mediated apoptosis or ripoptosis in the macrophages (A). In response to prolonged differentiation, macrophages have elevated levels of XIAP that inhibit all the caspases and $cFLIP_L$ that limits caspase 8 activation. Furthermore, the reduced activation of p38 MAPK and MK2 in macrophages with prolonged differentiation limits RipK1 phosphorylation and consequent ripoptosome complex formation. All these mechanisms culminates into reduced caspase activation and increased cell death resistance of the macrophages in response to ripoptosome signaling.

7.0. REFERENCES

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APPENDIX

Impact of SMAC mimetic, BV6 on macrophages at D5 and D12 of differentiation

BMDMs isolated at day 5 and day 12 of differentiation were subjected to BV6 treatment and the cell viability was assessed by MTT assay. Similar to BP treatment, D12 macrophages displayed significant resistance to BV6 induced cell death that was completely rescued by Nec-1 (Fig. I).

Role of the IFNAR1 and adaptor proteins MyD88 and TRIF in ripoptosome signaling

We investigated the downstream signaling mechanisms that are responsible for BP-induced cell death. We looked at the role of the IRNAR1 signaling and the adaptor proteins MyD88 and TRIF post BP treatment. BMDMs from WT, IFNAR1-/-, MyD88-/- and TRIF-/- mice were treated with BP on day 5 of differentiation and cell viability was assessed via MTT assay. IRNAR-/- and MyD88-/- macrophages were as susceptible as WT macrophages to cell death following BP treatment macrophages (Fig. IIA, B). On the other hand TRIF-/- macrophages seemed to be slightly resistant in comparison to WT macrophages (Fig. IIC). These data indicate that the adaptor protein TRIF has a minor role in ripoptosome-induced cell death, and IFNAR1 and MyD88 does not have any role.

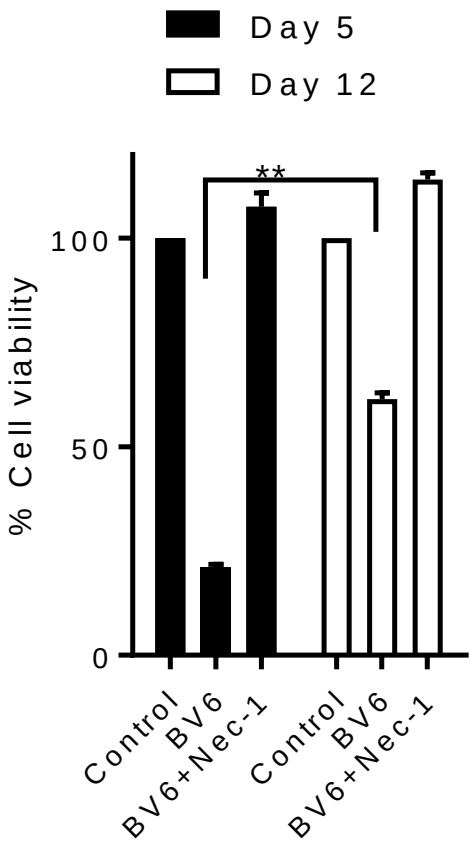

Figure I. Prolonged differentiation of macrophages develop resistance to BV6 induced cell death. BMDMs (at day 5 and day 12 of growth) from WT (C57BL/6) mice were treated with SMAC mimetic, BV6 (10μM) with and without necrostatin-1 (Nec-1) (10μM). After 24 hours incubation, the cell viability was measured by MTT assay. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

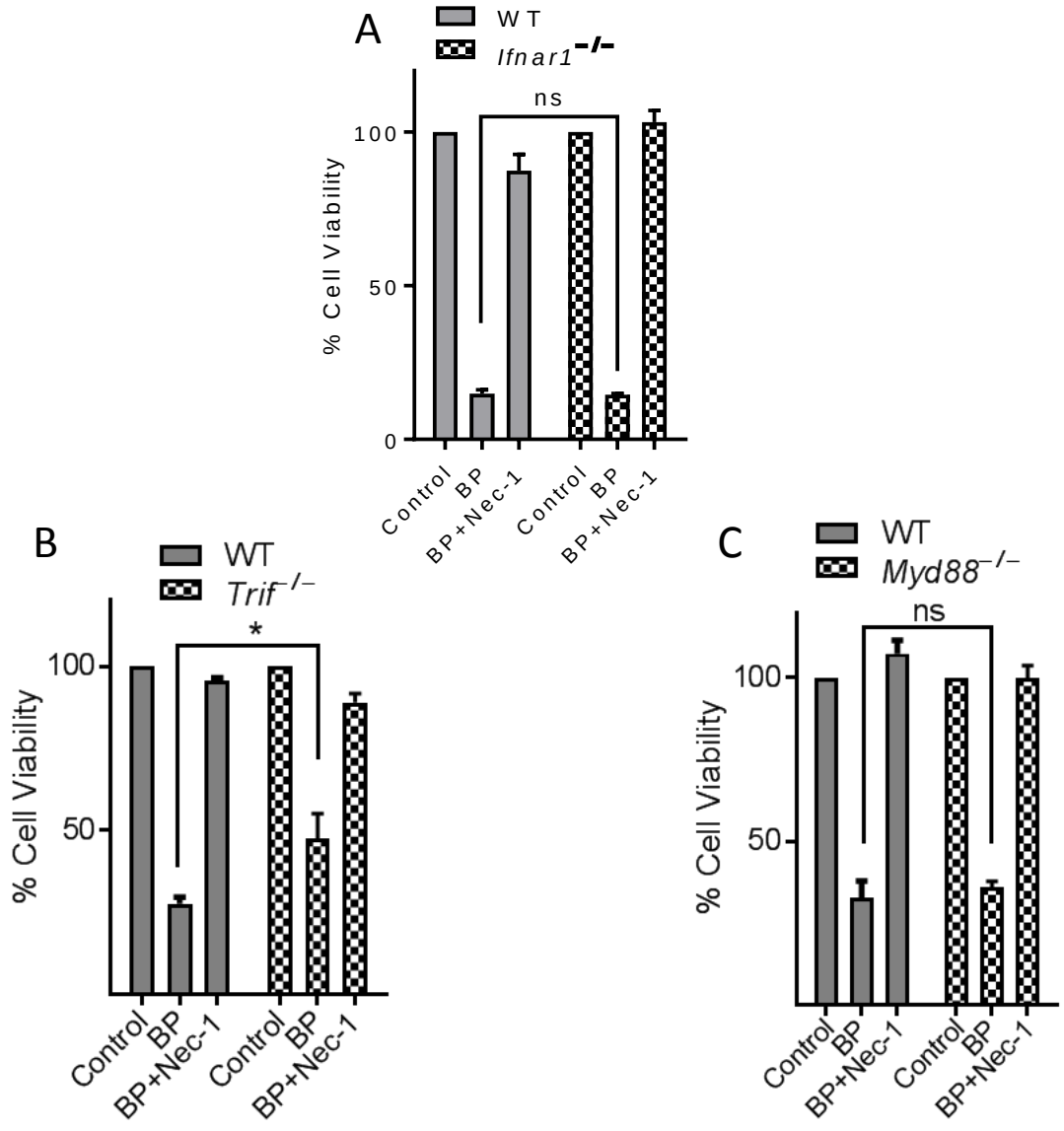

Figure II. BP induced death is independent on IFNAR1 and MyD88, while partially dependent on TRIF. . D5 BMDMs from WT (C57BL/6), IFNAR-/- (A), TRIF-/- (B) and MyD88-/- (C) mice were treated with Birinapant (10µM) with and without necrostatin-1 (10µM). After 24 hours incubation, the cell viability was measured by MTT assay. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

LPS+BV6 treatment does not overcome the resistance of macrophages induced by BV6

BMDMs isolated at day 5 and day 12 of differentiation were subjected to LPS+BV6 treatment and the cell viability was assessed by MTT assay. Similar to BV6 treatment, D12 macrophages displayed significant resistance to LPS+BV6 induced cell death (Fig. III). Unlike BV6 induced cell death, LPS+BV6 induced cell death was partially rescued by Nec-1, suggesting the cell death mechanism to be different than BV6 induced cell death.

Role of the RipK3, IFNAR1 and TNF-R signaling in TLR induced necroptosis

We evaluated the role of RipK3, IFNAR1 and TNF-R in LPS+zVAD induced necroptosis. BMDMs were generated from WT, RipK3-/-, IFNAR1-/- and TNF-R1/2-/- mice. On day 5 of differentiation, macrophages were treated with LPS+zVAD with and without Nec-1 and the cell viability was measured at 24 hours by MTT assay. As expected WT macrophages were susceptible to LPS+zVAD induced cell death, and RipK3-/- macrophages completely resistant (Fig. IVA). Interestingly, macrophages that are deficient in IFNAR showed significant resistance to cell death induced by LPS+zVAD (Fig. IVB) while TNF-R1/2-/- macrophages were highly susceptible to cell death (Fig. IVB). Taken together these results indicate that, TLR4 mediated necroptosis in macrophages is dependent on RipK3 and this form of cell death is promoted by IFNAR1 signaling. Also unlike BP induced ripoptosis and necroptosis, LPS+zVAD induced necroptosis is not dependent on TNF-R signaling.

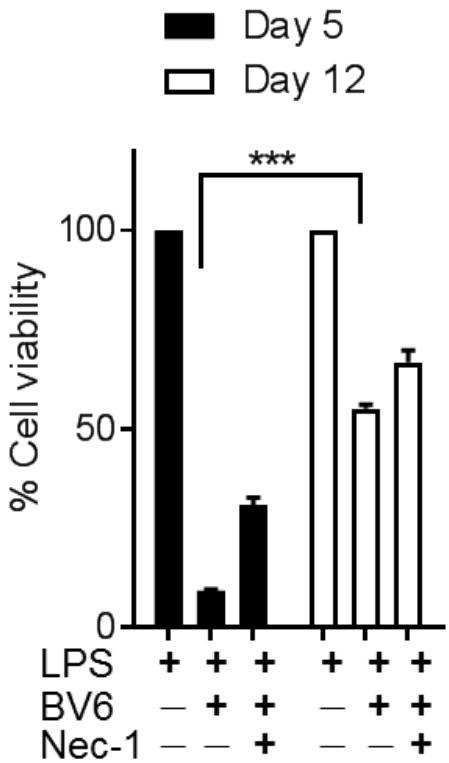

Figure III. Addition of LPS doesn't rescue the cell death induced by BV6. BMDMs (at day 5 and day 12 of growth) from WT (C57BL/6) mice were treated with LPS (1ng/ml) and SMAC mimetic, BV6 (10µM) with and without necrostatin-1 (Nec-1) (10µM). After 24 hours incubation, the cell viability was measured by MTT assay. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

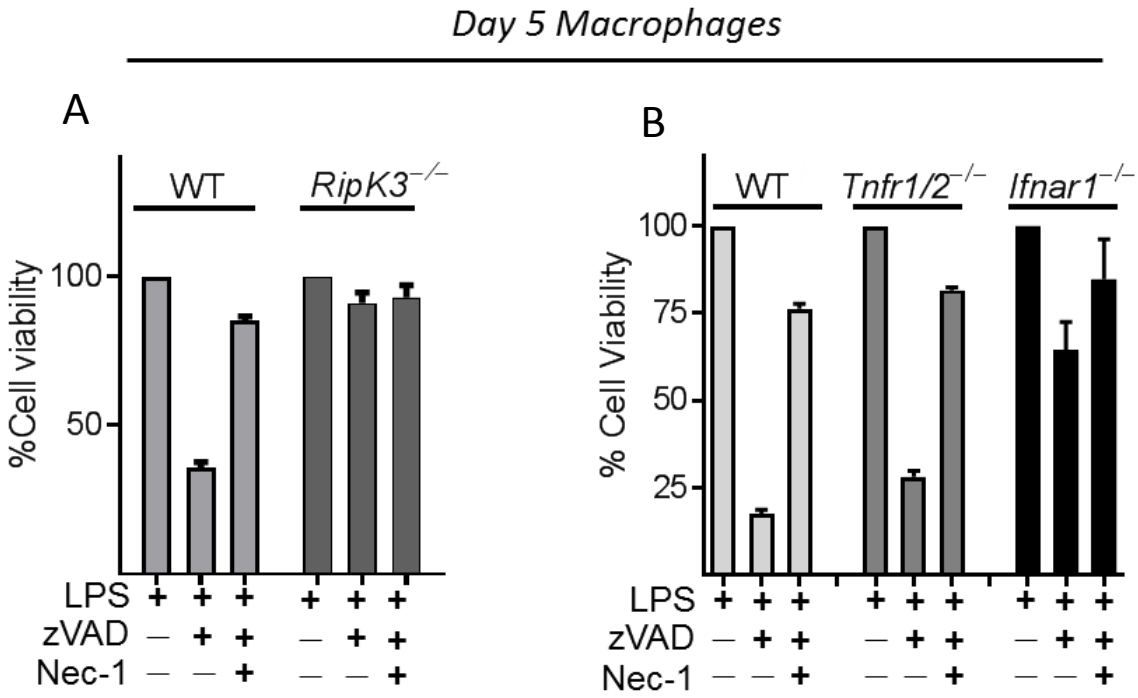

Figure IV. LPS+zVAD induced death is dependent on RipK3 and IFNAR1 while independent of TNFR. . D5 BMDMs from WT (C57BL/6), RipK3-/- (A), TNF-R1/2-/- and IFNAR-/- (B) mice were treated with LPS (1ng/ml) and zVAD (50µM) with and without necrostatin-1 (10µM). After 24 hours incubation, the cell viability was measured by MTT assay. Graphs show the percentage of viable cells ±SEM relative to control. All experiments were done at least in triplicates in mice aged 6-10 weeks. Statistical analyses done by unpaired students t-test *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; NS, not significant.

CURRICULUM VITAE

RIJAL, DIKCHHA

ACADEMIC INFORMATION

Master of Science in Microbiology and Immunology **2015 - 2017**
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa
(Major: Immunology)

Master of Science (82.80%) **2012**
Central Department of Microbiology
Tribhuvan University, Kathmandu, Nepal
(*Major: Medical Microbiology; Minors: Genetics, Biochemistry, Biotechnology, Epidemiology, Immunology, Biostatistics, Instrumentation, Anatomy and Physiology)*

Bachelor of Science (76.21%) **2007**
Central Campus of Science and Technology
Tribhuvan University, Nepal
(Major: Microbiology; Minors: Chemistry, Botany, Biostatistics, and Research Methodology)

Proficiency Certificate in Science (71.14%) **2003**
Central Campus of Science and Technology, Tribhuvan University, Dharan
(Major: Biology and Physics; Minors: Chemistry, Mathematics, and English)

RESEARCH EXPERIENCE

Graduate student – MSc. (Immunology) **2015**
University of Ottawa
Pursued research entitled "Cell death signaling complexes during macrophage differentiation".

The research project includes tasks such as:

- In vivo CFU and host survival experiments with *Salmonella enterica* Typhimurium infection.
- In vitro cell death and cytokine expression of murine macrophages.
- Flow cytometry analysis of macrophages at different stages of differentiation.
- Western blot analysis for protein detection

- ELISA for cytokines measurement
- qRT-PCR for measuring mRNA
- Co-Immunoprecipitation of interacting proteins
- Transfection
- Data analysis using Graphpad prism 7.

Thesis supervisor **2013**

Universal Science College

- Supervised undergraduate students in their thesis entitled "Isolation, characterization and antibiotic susceptibility test of gram negative uro-pathogens".
- Assessed the data entry, data interpretation and the results.

Medical Microbiology student **2010**

Tribhuvan University

- Researched on "Prevalence of *Pseudomonas aeruginosa* among the indoor patients of National Institute of Neurological and Allied Sciences and its antibiotic susceptibility profile".
- Handled the clinical specimens and performed the isolation, characterization and identification of the pathogens with the appropriate testing.
- Performed antibiotic susceptibity tests and determined the number of multi-drug resistant bacteria.
- Analyzed data using software such as SPSS 16

Volunteer **2010**

National Institute of Neurological and Allied Sciences

- Handled all the clinical specimens of the hospital and did the routine microbiological tests.
- Prepared the culture and biochemical media on regular basis and monitored the laboratory environment.
- Assisted the microbiologist of the hospital in data entry and preparation of reports.

PUBLICATIONS

- Contributing author- "K45A mutation of RIPK1 results in poor necroptosis and cytokine signaling in macrophages which impacts inflammatory responses in vivo" published in "Cell death and differentiation" on June 3, 2016.

- First author-"Prevalence of Pseudomonas aeruginasa among the indoor patients of National Institute of Neurological and Allied Sciences and its antibiotic susceptibility profile" – Published in the journal of Tribhuvan University, Central Department of Microbiology Kathmandu, Nepal on 2014.

ATTENDED CONFERENCE

Canadian Society for Immunology (CSI) Conference, Ottawa (2016)

- Active participation and Poster presentation.

ACADEMIC APPOINTMENTS

Lecturer **2011-2014**

Department of Microbiology

Universal Science College, Kathmandu, Nepal

- Facilitated theoretical and practical classes to undergraduate students.
- Supervised undergraduate students in their academic thesis through proper organization of research materials and being easily accessible.
- Successfully conducted periodic seminars, orientation sessions and workshops ensuring active participation of faculty members, students and external agencies.
- Evaluated periodic academic assessment of students.

Science teacher **2005-2006**

Sunakhari English Boarding School, Dharan, Nepal

- Managed a well-disciplined class by proper interaction, counseling and instruction to students.
- Conducted periodic academic evaluations of students with precision, along with dissemination of results to students and parents in a supportive environment.
- Motivated teachers or deliberation of class in an interactive environment to encourage student participation.

AWARDS

- Awarded "Admission Scholarship" at the University of Ottawa for securing higher than CGPA 8.0 in MSc. at Tribhuvan University.
- Received stipend from Tribhuvan University in 2007 for scoring the highest marks among the female students.
- Awarded for securing the second highest marks among the batch of Microbiology in 2007 by Nepal Academy of Science and Technology.
- Received an award of 'Certificate of Merit' in BSc (top scorer in Microbiology – 2004/2007) from Central Campus of Technology, Dharan, Nepal.
- Received an award of 'Certificate of Merit' in ISc. (Top scorer – 2001/2003) from Central Campus of Technology, Dharan, Nepal.

ATTENDED TRAININGS AND SEMINARS

- Attended training on WHMIS, Lab Safety Training, Worker Health and Safety Awareness, National Institutional Animal User Training (NIAUT), Principles of Biosafety, Violence Prevention, Respect in the workplace, qRT-PCR and Quantitative Western blotting in University of Ottawa.
- Actively participated in 'The Fifth National Conference on Science and Technology", Kathmandu, Nepal, on November 10-12, 2008, organized by NAST.
- Participated in 15-hour comprehensive training on 'Bioinformatics, its basics, scopes in different fields and applications in Microbiology and Genetics', on August 18-22, 2008.