Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Marie Namtvedt Holter-Sørensen

SMAC-mimetic induced cell death in LPS-pretreated human macrophages

Master's thesis in Biotechnology
Supervisor: Kristian Kobbenes Starheim, IKOM/CEMIR
Co-supervisor: Ingrid Nyhus Moen, IKOM/CEMIR
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Abstract

Inflammation is a biological response in body tissue due to harmful stimuli. In order to maintain homeostasis in the body, immune cells need to be tightly regulated. Dysregulated activity of the immune cells called macrophages result in chronic inflammation and aberrant pathogen response. The receptor-interacting serine-threonine protein kinase 1 (RIPK1) is a central regulator of inflammation and cell death in macrophages. RIPK1 is tightly regulated by ubiquitination of cellular inhibitor of apoptosis protein (cIAP) 1 and 2 which acts as a switch between cell survival and death. SMAC-mimetics (SMs) are small molecules which specifically inhibit IAPs and are under clinical trials for treatment of cancer. Inhibition of the IAPs results in deubiquitylation of the kinase and induction of RIPK1-dependent cell death. Knowledge about SM-induced cell death is necessary in order to understand how it can be used for treatment of diseases. In this project we investigate how SM works in macrophages under different conditions. A more precise understanding of these mechanisms can lead to novel therapeutic approaches for treating inflammatory diseases.

Lipopolysaccharide (LPS) is a cell wall component of gram-negative bacteria. It binds to the host receptor TLR4 to activate first-line immune responses and prepare the immune system for subsequent responses. Pretreatment by LPS in healthy human macrophages increased the cytotoxicity of birinapant-induced cell death *in vitro*. We did not observe the same effect with LCL-161, another SM, suggesting that birinapant was more potent to induce cell death. In addition to LPS, pretreatment by TNF-α was also tested since both activate NF-κB signaling. TNF-α-pretreatment did not potentiate birinapant-induced cell death suggesting that LPS acts specific in macrophages. In addition, some production of IL-1β was observed in birinapant-stimulated macrophages pretreated with LPS. Stimulation by LPS for 6 hours also increased the levels of the anti-apoptotic protein c-FLIP in the cells. Immunoblot analysis showed increased protein levels of caspase-3, caspase-8 and GSDMD p43 cleavage products when birinapant-stimulated macrophages were pretreated with LPS. Despise high c-FLIP levels, caspase-8 seemed to still be able to execute cell death, which most likely was apoptosis. In conclusion, we here showed that LPS-pretreatment potentiated birinapant cytotoxicity in human macrophages and short pretreatment was sufficient.

Sammendrag

Betennelse er en biologisk respons i kroppsvevet forårsaket av skadelig stimuli. For å opprettholde homeostase i kroppen er det viktig å nøye regulere immuncellene. Dysregulert aktivitet av en immuncelletype som heter makrofager kan resultere i kronisk betennelse og avvikende patogenrespons. Reseptor-interagerende serin-threonin protein kinase 1 (RIPK1) er sentral for reguleringen av betennelse og celledød i makrofager. RIPK1 er nøye regulert av ubiquitinering av cellulære hemmere av apoptose protein (cIAP) 1 og 2. Disse fungerer som en bryter mellom celleoverlevelse og celledød. SMAC-etterlignere (SMer) er små molekyler som spesifikt hemmer IAPene og er under klinisk testing som kreftbehandling. Dersom IAPene blir hemmet fører dette til deubiquitinering av kinasen og induserer RIPK1-avhengig celledød. Det er nyttig å ha kunnskap om SM-indusert celledød for å forstå hvordan det kan anvendes i behandling av sykdommer. I dette prosjektet undersøkte vi hvordan SM virker på makrofager under ulike betingelser. En mer presis forståelse av disse mekanismene kan føre til nyskapende terapier for behandling av betennelsessykdommer.

Lipopolysakkarid (LPS) er en bestanddel av celleveggen til gram-negative bakterier. Den binder seg til vertsreseptoren TLR4 for å aktivere førstelinjen i kroppens immunforsvar og forbereder immunsystemet på påfølgende responser. Forbehandling med LPS i humane makrofager økte cytotoksisiteten til birinapant-indusert celledød *in vitro*. Vi observerte ikke samme effekt med LCL-161, en annen SM. Dette antyder at birinapant var mer potent til å indusere celledød enn LCL-161. I tillegg til å teste LPS, ble også TNF-α forbehandling testet siden begge aktiverer NF-κB signalisering. Forbehandling med TNF-α potenserte ikke birinapant-indusert celledød. Dette antyder at LPS har en spesifikk effekt i makrofager. I tillegg ble det observert IL-1β produksjon i birinapant stimulerte makrofager som ble forbehandlet med LPS. Stimulering med LPS i 6 timer økte nivåene av det anti-apoptotiske proteinet c-FLIP i cellene. Immunoblott-analyser viste økt protein nivåer av caspase-3, caspase-8 og GSDMD p43 kløyvingsprodukter når birinapant-stimulerte makrofager fikk LPS forbehandling. Til tross for høye nivåer av c-FLIP i cellen, ser det ut til at caspase-8 fremdeles var i stand til å utføre celledød. Denne celledøden var mest sannsynlig apoptose. For å oppsummere viser vi i dette arbeidet at LPS forbehandling potenserer birinpant sin cytotoksisitet i humane makrofager, og kort forbehandling var tilstrekkelig.

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Marie Namtvedt Holter-Sørensen

Trondheim, May 2021

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Abbreviations

AIM2	Absent in melanoma 2
ALR	AIM2-like receptor
AP-1	Activator protein-1
ASC	Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain
ATP	Adenosine triphosphate
BIR	Baculoviral IAP repeat
CARD	Caspase recruitment domain
CD	Cluster of differentiation protein
CD40L	Cluster of differentiation 40 ligand
CFLAR	CASP8 and FADD-like apoptosis regulator
c-FLIP	Cellular FLICE inhibitory protein
cIAP	Cellular inhibitor of apoptosis protein
CSF-1	Colony-stimulating factor 1
CSF-1R	Colony-stimulating factor 1 receptor
CYLD	Cylindromatosis, CYLD lysine 63 deubiquitinase
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DED	Death-effector domain
DISC	Death-inducing signaling complex
DUB	Deubiquinating enzyme
ERK	Extracellular signal-regulated kinase
FADD	FAS associated via death domain
GSDMD	Gasdermin D

HMGB1	High mobility group box 1
HSC	Hematopoietic stem cell
IBM	IAP-binding motif
IAP	Inhibitor of apoptosis protein
IFN	Interferon
IκB	Inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
IRF	Interferon-regulatory factor
JNK	c-Jun NH_2-terminal kinase
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin chain assembly complex
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-colony-stimulating factor
MD	Myeloid differentiation
MLKL	Mixed lineage kinase domain-like protein
MyD88	Myeloid differentiation factor 88
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor κ-light chain enhancer of activated B-cells
NIK	NF-κB-inducing kinase
NLR	Nucleotide-binding leucine-rich repeat receptor
NLRP3	NLR family pyrin domain containing 3
PAMP	Pathogen-associated molecular pattern
RHIM	RIP homotypic interaction motif
RIPK	Receptor-interacting serine-threonine protein kinase

ROS	Reactive oxygen species
PRR	Pattern recognition receptor
SMAC	Second mitochondrial activator of caspases
SM	SMAC-mimetic
STAT	Signal transducer and activator of transcription
TAB	TAK1-binding protein
TAK1	Transforming growth factor β-activated kinase 1
TAM	Tumor-associated macrophage
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR1-associated death domain protein
TRAF	TNF receptor associating factor
TRAM	TRIF-related adaptor molecule
TRIF	Toll/interleukin-1 receptor domain-containing adaptor-inducing interferon-β
Ub	Ubiquitin
XIAP	X-linked inhibitor of apoptosis protein

1 Introduction

1.1 The monocytic linage

1.1.1 The monocyte

Monocytes are a type of white blood cell that play an essential part in the innate immune system. Originating from hematopoietic stem cells (HSCs) in the bone marrow, the cells differentiate through a series of stages to become monocytes. They are found in the spleen and bone marrow and circulate in the blood. Morphological features of monocytes include an irregular cell shape with a kidney-shaped nucleus, a high cytoplasm-to-nucleus ratio, and cytoplasmic vesicles. Human peripheral blood monocytes are heterogeneous and categorized into three subtypes based on their expression of different Cluster of Differentiation proteins (CD) on their surface. The subgroups are distinguished based on the composition of CD14 and CD16 surface proteins. Most monocytes (~85%) belong to the subgroup classical monocytes, expressing CD14 and not CD16 [1, 2]. Monocytes are crucial for innate immunity as they mediate host defense against pathogens by differentiating into macrophages and dendritic cells (DCs), when exposed to microbial molecules or stimulated with cytokines [3].

1.1.2 Macrophages and polarization

Macrophages are multifunctional cells important for tissue development and repair, eliminating pathogens and clearing apoptotic cells [4, 5]. The importance of macrophages is reflected by their presence in different tissues where they exhibit functional and anatomical diversity. Macrophages present in the skin are Langerhans cells, while other macrophages called osteoclasts reside in the bone. When tissue homeostasis is in steady-state tissue-resident macrophages, such as alveolar macrophages in the lung, have intrinsic anti-inflammatory functions to prevent persistent inflammation [6]. During increased caloric intake, macrophages of the liver called Kupffer cells regulate metabolic adaptions of hepatocytes. In response to environmental changes or tissue physiology, macrophages change phenotype and functional state to adapt to different challenges. And their ability to distinguish between self and non-self is essential for this [7].

Colony-stimulating factor 1 (CSF-1, previously known as macrophage-CSF, M-CSF) is a growth factor essential for macrophage differentiation. Mature monocytes express CSF-1 receptor (CSF-1R), which binds CSF-1 and cytokines [2, 4]. When infection occurs, circulating monocytes move from the bloodstream to the inflammatory tissue. Here the monocytes differentiate into peripheral mononuclear phagocytes, such as macrophages and DCs [2, 3].

The phenotype of macrophages is determined by polarization in response to different stimuli [5]. Monocytes stimulated with CSF-1 is the primary regulator of macrophage differentiation (Figure 1.1) [8]. Macrophages change their phenotype depending on the microenvironment and this process is dynamic [5]. Earlier, these cells have been categorized as pro-inflammatory M1 and anti-inflammatory M2 macrophages based on the direction of the polarization. Now it is more common to regard the nomenclature of macrophages based on which cytokine or other modulators they are stimulated with, such as M(LPS), M(TNF-α) and M(IL-4). This is grounded in the fact that M1/M2 categorization does not represent the natural condition in the body, as stably defined groups do not exist here. Rather, the macrophages respond to a combination of factors in their present environment and should be referred to according to these stimuli [9].

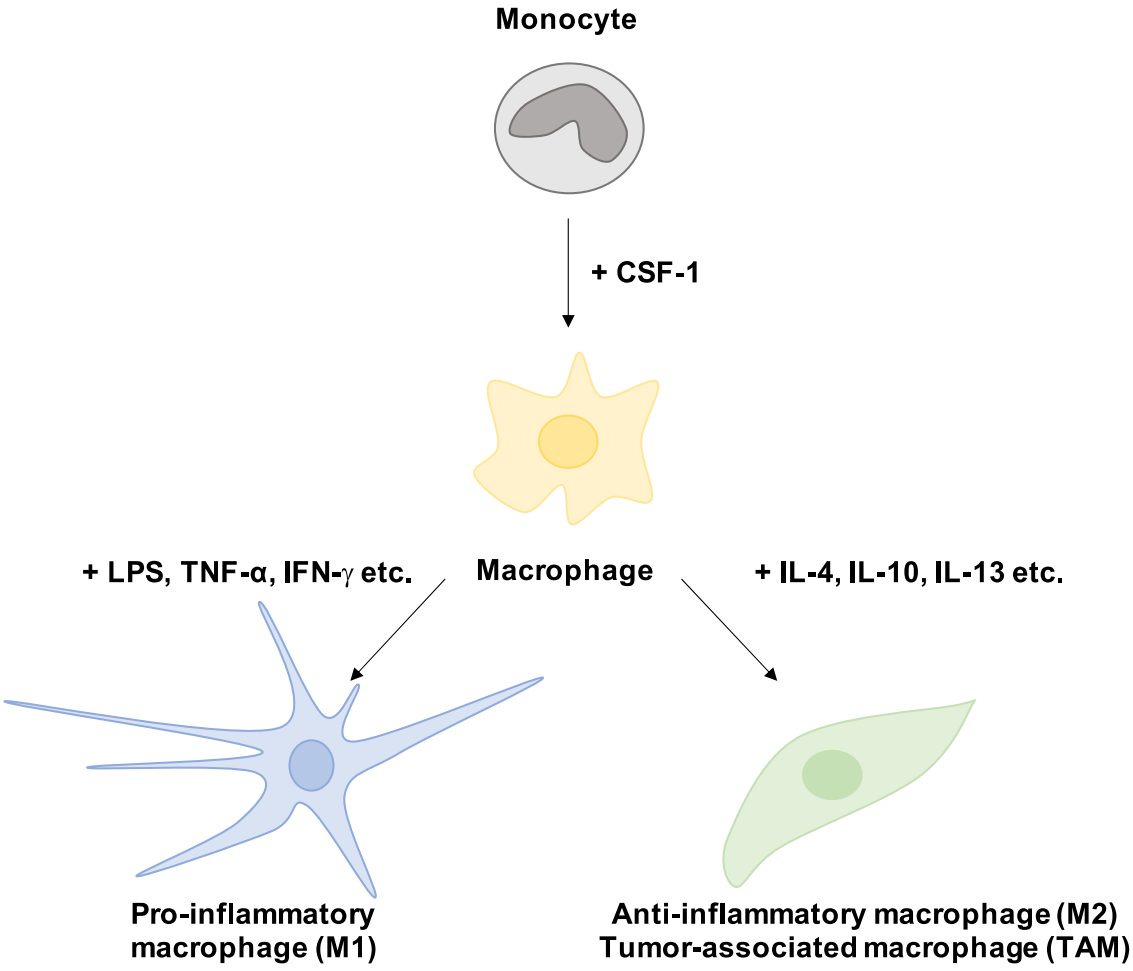


Figure 1.1 Macrophage polarization. Human monocytes are dependent on colony stimulating factor-1 (CSF-1) to differentiate into macrophages. A pro-inflammatory phenotype is obtained by treating the macrophages with stimulants, such as LPS, TNF-α and IFN-γ. Polarization towards an anti-inflammatory phenotype is promoted by cytokines, including IL-4, IL-10 and IL-13. Modified from [10].

Pro-inflammatory macrophages, earlier referred to as M1 or classically activated macrophages, are important for the host defense against infection [5]. Macrophages polarize into this subtype when exposed to specific cytokines, such as tumor necrosis factor (TNF)-α or interferon (IFN)-γ. Alternatively, pathogens can stimulate these cells to polarize into this phenotype through activation of toll-like receptors (TLRs). An example is lipopolysaccharide (LPS) from gram-negative bacteria stimulates these cells to polarize into this phenotype [1]. LPS is a type of pathogen-associated molecular pattern (PAMP) which macrophages recognize with their TLR4. Activation of TLR4 leads to signaling via nuclear factor-kappa B (NF-κB) resulting in production of inflammatory cytokines, such as TNF-α and interleukin 6 (IL-6) [1, 5, 11]. Prolonged or chronic activation of the pro-inflammatory macrophages can damage host tissue and lead to inflammatory diseases [5, 11, 12].

Anti-inflammatory macrophages, previously referred to as M2 or alternatively activated macrophages, are necessary for protecting the host against excessive injury. These macrophages provide tissue remodeling, and promote tissue healing and growth [5, 11]. Polarization towards an anti-inflammatory phenotype requires cytokines such as IL-4, IL-10 and IL-13. These cytokines activate signaling through STAT3/STAT6 (signal transducer and activator of transcription 3 or 6). This results in the release of transforming growth factor (TGF)-β and IL-10, which promote dampening of immune responses [5, 8, 12]. The tumor-associated macrophages (TAMs) show a similar phenotype to anti-inflammatory macrophages. However, TAMs are essential for cancer formation, angiogenesis, dampers the immune recognition of tumor cells and promote immune evasion [4, 5, 13].

The classification of M1 and M2 polarization of macrophages only represent the terminals of a continuum, and it is a dynamic process. The M1/M2 crosstalk is mediated by activation of STAT1 and STAT3/STAT6 signaling. NF-κB, interferon-regulatory factor (IRFs) and activator protein (AP)-1 are some key transcription factors involved in macrophage polarization [5]. In sepsis, NF-κB activation drives macrophages to its M1 phenotype to induce a response to inflammation but later polarize to the M2 phenotype by inhibiting NF-κB to exhibiting anti-inflammatory features. Populations of macrophages in disease settings can also have mixed phenotypes. Little is known about the exact switch that manage the macrophage phenotype and the molecular networks involved [12].

1.2 NF-κB signaling

Nuclear factor κ-light chain enhancer of activated B-cells (NF-κB) is a family of transcription factors regulating many cellular functions such as in inflammation and immune cell survival [14]. It is a central transcription mediator of pro-inflammatory macrophages as it is essential for induction of inflammatory genes [15]. A wide range of stimuli activate NF-κB, including LPS and cytokines [16]. The main function of this transcription factor is to maintain and initiate inflammatory conditions through the innate and adaptive immunity upon autoimmune and pathogenic stimuli [17]. NF-κB binds to DNA sites to express over 150 target genes encoding proteins, including cytokines such as IL-6 and TNF-α [16, 18]. The transcription factor genes can also inhibit pro-apoptotic signaling pathways by inducing factors directly targeting the caspase cascade, such as cellular FLICE inhibitory protein (c-FLIP) and inhibitor of apoptosis proteins (IAPs) [19]. The NF-κB family consists of RelA (p65), p50/p105, RelB, p52/p100 and c-Rel, which form either homodimers or heterodimers [14, 16, 18]. Dysregulation of NF-κB is involved in several inflammatory diseases, such as multiple sclerosis (MS) and rheumatoid arthritis (RA), and is therefore an attractive target for anti-inflammatory therapies [15].

Two signaling pathways lead to the activation of NF-κB: the canonical (classical) and the non-canonical (alternative) pathways (Figure 1.2) [18]. The canonical pathway is rapid and is involved in the innate immune response. In contrast, the non-canonical pathway responds slower but provides a prolonged NF-κB activity. It also ensures an effective immune response as it is associated with the development of the lymphoid organs [19, 20].

Several receptors activate the canonical pathway, including TNF receptors (TNFRs) and TLR4 (Figure 1.2, A) [14, 18]. Upon activation by TNF-α and LPS, respectively, the receptors recruit a TNF receptor associating factor (TRAF). TRAF recruits receptor-interacting serine-threonine protein kinase 1 (RIPK1) and cellular inhibitor of apoptosis protein (cIAP) 1 and 2 to the membrane-bound receptor complex. RIPK1 and cIAP1/2 will be further discussed later. The transforming growth factor β-activated kinase 1 (TAK1)-binding protein (TAB) 2 recruits TAK1 to TRAF [21, 22, 23]. Inhibitor of NF-κB (IκB) consists of two catalytic subunits, IκB kinase (IKK)β and IKKα, and the regulatory subunit IKKγ/NEMO, comprising the IKK complex. The kinase protein TAK1 directly phosphorylates IKKβ [20, 23]. Active IKKβ phosphorylates IκBα bound to the RelA/p50 heterodimer. Proteases polyubiquitinate and degrade IκBα resulting in

the release of the heterodimer, which translocates to the nucleus to induce transcription of target genes [19, 20].

While the canonical pathway depends on the degradation of IκB to release NF-κB, the non-canonical pathway has a different mechanism (Figure 1.2, B). Cytokines belonging to the TNF family, such as CD40 ligand (CD40L), activate this pathway [18]. When binding to the TNFR, NF-κB-inducing kinase (NIK) indirectly phosphorylate p100 through the kinase IKKα, leading to the processing of p100. Phosphorylated and ubiquitinated p100 lead to the generation of p52. The heterodimer RelB/p52 translocates to the nucleus to target genes for transcription [24, 25].

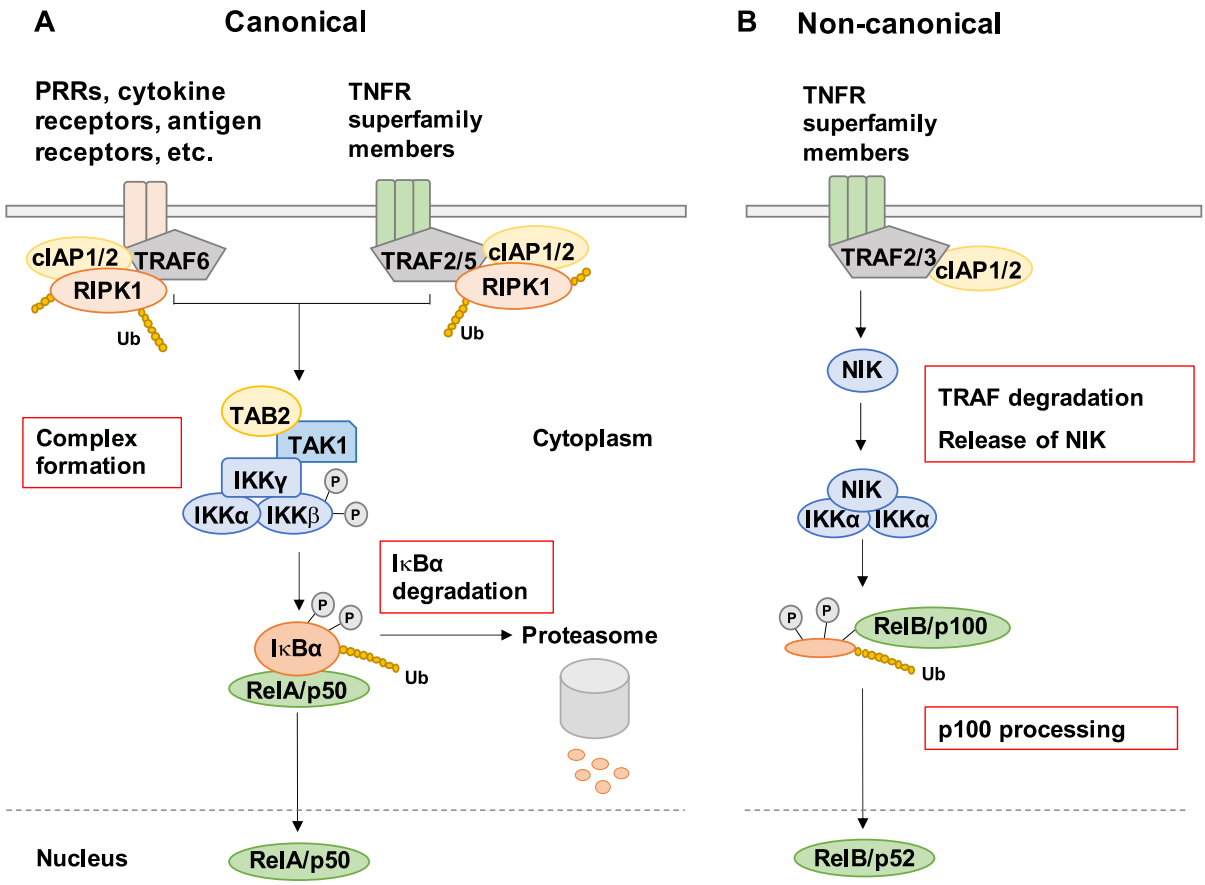


Figure 1.2 Canonical and non-canonical NF-κB signaling. A, B. (**A**) The canonical NF-κB pathway is induced by LPS and several types of cytokines, including TNF-α. Activation of TLR4 and TNFR1 result in signaling which involves induction of the IKK complex by TAK1. IκBα gets phosphorylated by IKKβ and ubiquitinated for degradation by the proteasome. This results in the release of RelA/p50 heterodimer which translocates to the nucleus. (**B**) The non-canonical NF-κB pathway activates TNFR superfamily members, such as CD40. Cytokines from the TNF family, including CD40L are involved in the receptor activation. Signaling involves processing of p100 by NIK and IKKα. This results in the generation of p52, and RelB/p52 heterodimer can translocate to the nucleus. Modified from [14, 24].

1.3 The MAPK family

Mitogen-activated protein kinases (MAPKs) are a mammalian family of serine-threonine kinases. They are often activated together with NF-κB downstream of TLR4, TNFR1 and TAK1. Therefore, activation of MAPKs results in some of the same responses as NF-κB, such as producing pro-inflammatory cytokines. MAPKs are responsible for regulating cellular activities, including differentiation, proliferation, survival and death. The family consists of extracellular signal-regulated kinase (ERK), p38 and c-Jun NH_2-terminal kinase (JNK). Activation of each pathway results in a signaling cascade including at least three components: a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K) and a MAPK. The pathways are activated by intracellular and extracellular stimuli, such as cytokines, hormones and cellular stressors [26, 27].

Stimulation of TNFR or TLR4 result in RIPK1-mediated activation of TAK1. TAK1 is a MAP3K and phosphorylates a downstream MAP2K, which in turn phosphorylates the MAPKs ERK, p38 or JNK [28, 29]. Activated MAPKs phosphorylate substrate proteins, such as the transcription factors c-Jun, p53 and AP-1, regulating a variety of cellular activities. This includes inflammatory responses, proliferation and death. MAPK signaling is guided by scaffold proteins which consist of specific kinase components depending on the pathway induced [26]. TAK1-deficient cells will not activate JNK, p38 nor NF-κB when stimulating with TNF-α, IL-1 or TLR-ligands, such as LPS [28].

1.4 The RIPK1-pathway

Receptor-interacting serine-threonine protein kinase 1 (RIPK1) is a cytosolic protein kinase. It is involved in three signaling cascades: NF-κB/MAPK signaling, and apoptotic and necroptotic cell death [30]. The RIPK1-pathway is activated by pro-inflammatory cytokines, including TNF-α, IL-6, IL-1β and IFNs, and microbial stimuli such as LPS. As previously described, activation of TNFR or TLR4 results in the formation of the receptor complex which recruits RIPK1 [31]. This results in activation of the transcription factors NF-κB and AP-1, and cell death [30]. RIPK1 is a fundamental mediator of the innate immune response because its activation can result in several cell death and inflammation outcomes. Dysregulation of RIPK1 can lead to detrimental consequences, such as tissue damage. Therefore, it needs strictly regulation to maintain control of normal homeostasis in tissues [31]. The main focus for this work will be on TLR4 signaling induced by LPS, as well as TNFR1-signaling [32].

1.4.1 RIPK1-activation of NF-κB and MAPK

RIPK1 is necessary for TNFR1 activation of NF-κB/MAPK. Activation of TNFR1 by TNF-α stimulates the adaptor protein TNFR1-associated death domain protein (TRADD). TRADD brings RIPK1 and TRAF2 together to form a membrane-bound complex. cIAP1 and cIAP2 are bound to TRAF2 which brings the complex to the intracellular domain of TNFR1. When RIPK1 is part of this receptor complex, ubiquitin enzymes including cIAPs can polyubiquitinate the kinase. The ubiquitination is central for whether the signaling results in cell survival or death. Modulators of the canonical NF-κB and MAPKs pathways, such as TAK1, can bind to this polyubiquitinated site. This results in survival and induce inflammatory responses as previously described (1.2) [30, 31, 32]. Recruitment of deubiquinating enzymes (DUBs) to the activated TNFR1-complex results in deubiquitylation of RIPK1 by these enzymes which induce cell death. [32, 33].

1.4.2 cIAP1 and 2 ubiquitinate RIPK1 to form a TNFR1-RIPK1 receptor complex

Ubiquitin (Ub) is a small protein, about 8 kDa, which covalently attach to a substrate protein. This process is called ubiquitination and involves three classes of enzymes: E1, E2, and E3 ubiquitin ligases. A ubiquitin-activating enzyme (E1) binds Ub with a thioester linkage before transferring the Ub to a ubiquitin-conjugating enzyme (E2). E2 consists of an active site cysteine, which the Ub binds to. Lastly, the mediation of Ub from E2 to a specific substrate protein is conducted by a ubiquitin ligase (E3). The substrate contains a lysine residue that binds to a C-terminal glycine at the Ub, and they form an isopeptide bond. Ub can also form a polyubiquitin chain consisting of Ub molecules conjugated to each other [32]. Ub-chains are often studied as tags for degradation via the proteasome or autophagy [34, 35]. In the RIPK1 signaling pathway the Ub-chains display another function: they perform as scaffolds to recruit and build multimeric signaling complexes [36]. The Ub molecule consists of seven internal lysine residues (such as K11, K48, and K63), and one of these can bind to one lysine residue of another Ub molecule. Alternatively, formation of linear ubiquitin linkage can be assembled if Ub conjugates to another Ub by attaching to its α-amino group of the N-terminal methionine [32].

Regulation of RIPK1 by ubiquitination and deubiquitylation mediates whether the signaling results in NF-κB/MAPK activation or cell death, respectively. Ubiquitinated RIPK1 performs as a scaffold and not a kinase. However, its kinase activity is activated in necroptosis and

sometimes during apoptosis [32]. Various lysine-linked polyubiquitin chains are involved in the scaffolding function of RIPK1. This includes K63-linked polyubiquitination which recruits factors needed to stabilize the TNFR complex to induce NF-κB and MAPKs signaling [37]. cIAP1 and cIAP2 mediate this ubiquitination [33]. Other ubiquitin ligases are also involved in the ubiquitination of RIPK1, such as the linear ubiquitin chain assembly complex (LUBAC) [32, 33].

cIAPs are ubiquitin ligases belonging to the IAP family. They are central regulators of cell death [32]. There are a total of eight members in the family. The cIAP are characterized by the presence of one or several Baculoviral IAP Repeats (BIRs) which indirectly bind to and inhibit caspases [38, 39]. Two of the members, cIAP1 and cIAP2, are recruited to inflammatory receptors, and are involved in inflammatory responses and extrinsic cell death regulation. By interacting with TRAF1 and TRAF2, cIAP1 and cIAP2 regulate TNFR1 signaling pathway [40, 41].

1.4.3 TLR4 activates RIPK1 via TRIF signaling

TLR4 is a pattern recognition receptor (PRR) belonging to the TLR family. The family consists of thirteen members recognizing PAMPs, such as bacteria. The activation of TLRs is important for the innate immunity as it results in inflammatory responses. Immune cells, including monocytes and macrophages, express TLR4. The plasma membrane anchored CD14 protein displayed on these immune cells and the myeloid differentiation (MD) factor-2 protein facilitate the binding of LPS to TLR4 [42].

Lipopolysaccharide (LPS) is a cell wall component of gram-negative bacteria [42]. LPS binding triggers TLR4 signaling via MyD88 (myeloid differentiation factor 88) and TRIF (Toll/IL-1 receptor homology domain (TIR)-containing adaptor inducing IFN-β) (Figure 1.3). Almost all TLRs use MyD88 to activate a rapid NF-κB/MAPK response. Signaling via the adaptor protein TRIF is unique for TLR4 and activates several pathways, including RIPK1. Endocytosis of TLR4 is needed for activation and recruitment of TRIF. TRIF-related adaptor molecule (TRAM) is required for TRIF recruitment to endosomal TLR4. Active TRIF recruits TRADD, TRAF6, cIAP1/2 and RIPK1 to the C-terminal RIP homotypic interaction motif where cIAP1/2 polyubiquitinate RIPK1. TAK1 binds to the polyubiquitin chain which results in activation of NF-κB and MAPK cascades, as previously described in 1.2 and 1.3. If RIPK1 is deubiquitylated the signaling will change to cell death [43, 44, 45].

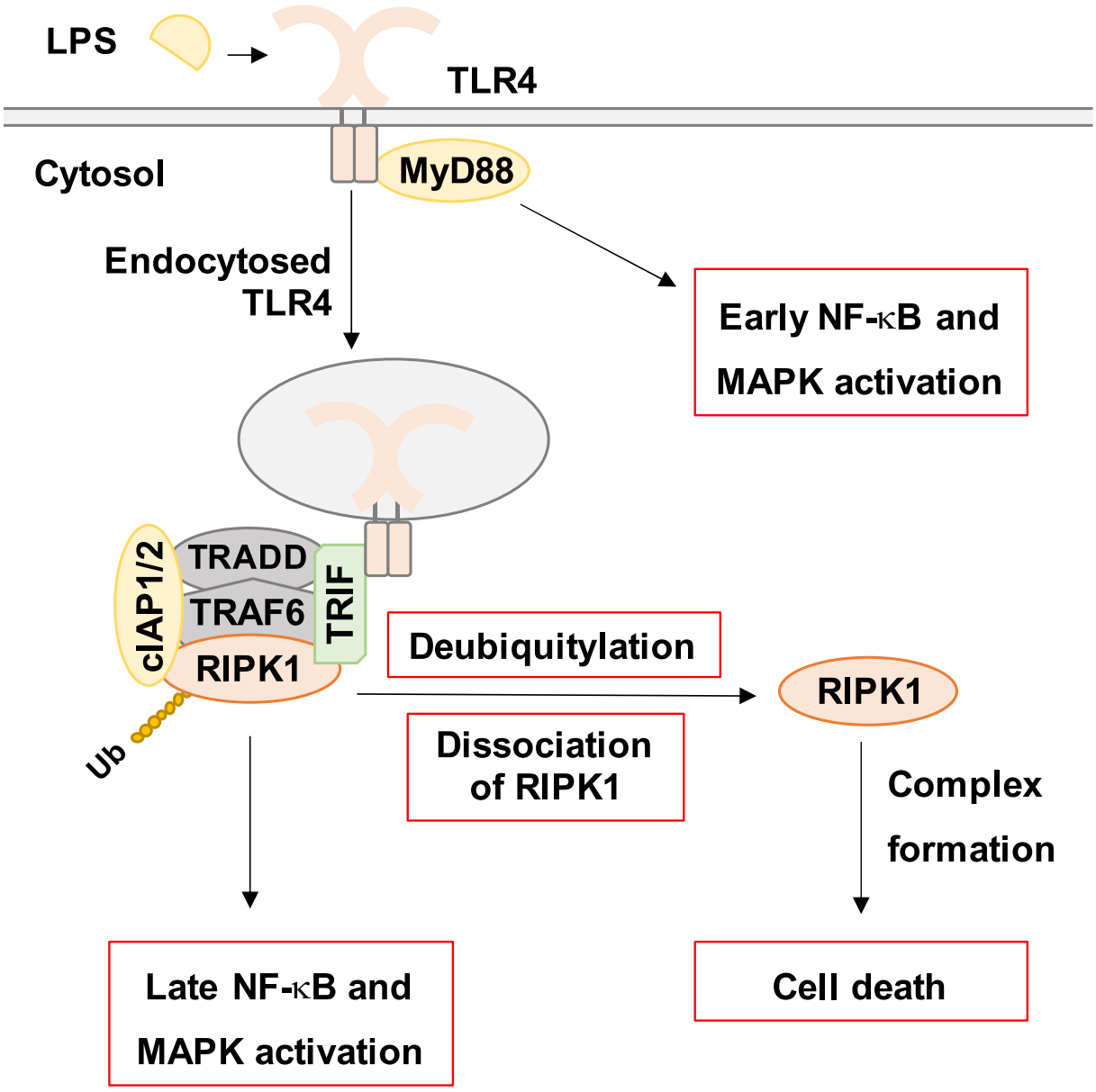


Figure 1.3 TLR4 activates RIPK1-dependent cell death via TRIF signaling. Stimulation with LPS activates TLR4. Two signaling pathways can be induced: MyD88 and TRIF. MyD88 binds to the intracellular domains of TLR4 activating early NF-κB and MAPK signaling. Activation of TRIF requires internalized endosomal TLR4. The receptor is endocytosed which activates TRIF. TRADD, TRAF6, cIAP1/2 and RIPK1 are recruited to TRIF. Polyubiquitination of RIPK1 results in late NF-κB and MAPK activation. Deubiquitylation of RIPK1 causes it to dissociate from the TLR4-bound complex into cytosol. Complex formation with other proteins results in RIPK1-mediated cell death. Modified from [42, 44].

1.5 RIPK1 coordinates PANoptosis

Deubiquitylation of RIPK1 changes its signaling from the NF-κB/MAPK pathways to cell death [32]. Several DUBs are responsible for deubiquitylation of RIPK1, including cylindromatosis (CYLD) and A20. Alternatively, the absence of ubiquitin ligases, such as cIAP1/2, leads to no ubiquitination of RIPK1 at the receptor complex, which also results in cell death activation [31, 46]. Deubiquitylation of RIPK1 causes the kinase to dissociate from the membrane-bound complex and construct death-inducing complexes in the cytosol [47]. The downstream signaling of RIPK1 results in caspase 8-dependent apoptosis. But under certain circumstances, other forms of cell death such as RIPK3-dependent necroptosis or inflammasome-dependent pyroptosis can be activated. The term PANoptosis (pyro/apo/necroptosis) has been coined to describe the extensive crosstalk between these three cell death forms [32, 37].

1.5.1 RIPK1-dependent apoptosis

Apoptosis is a type of cell death. An apoptotic cell is typically characterized by chromatin condensation, cytoplasmic shrinkage, plasma membrane blebbing, and small intact vesicles (apoptotic bodies) [48, 49, 50]. Apoptosis is immunologically silent, meaning that the cell does not cause inflammatory reactions at the tissue site. This is because no cellular constituents are released by the apoptotic cell to surrounding tissue, and if so, it is quickly phagocytosed by nearby cells [49, 50, 51].

Apoptosis can signal through the extrinsic or the intrinsic pathways. The extrinsic pathway receives its signal from the extracellular environment, such as the death receptor Fas (CD95), or inflammatory receptors such as TLR4 and TNFR1. In the absence of cIAPs or kinases, including TAK1, TNFR1 promotes another receptor complex consisting of RIPK1, FAS-associated death domain (FADD), and caspase-8 (Figure 1.4) [39, 51, 52]. A death-inducing signaling complex (DISC) forms when FADD connects to procaspase-8, and autocatalytic activation of the caspase is initiated. Caspase-8 activates the executioner caspases, such as caspase-3, which leads to apoptotic cell death [50].

Caspases are a family of cysteine proteases that regulate processes such as apoptosis and inflammation [41]. They can be divided into initiator, executioner and inflammatory caspases. The initiator caspases include caspase-2, -8, -9 and -10 and become active when interacting with death-effector domains (DEDs) or caspase recruitment domains (CARDs) of the extrinsic and intrinsic pathways [38]. Activation of the initiator caspases results in formation of multimers,

and cleavage of their partners occurs. The initiator caspases have a critical role in triggering the downstream executioner caspases, including caspase-3, -6 and -7. These caspases are inactive multimers until the initiator caspases cleave their protease domain which results in rearrangement of the executioner caspases. The executioner caspases then go on to cleave factors such as the plasma membrane channel pannexin-1 leading to the execution of apoptosis [50, 52, 53]. Inflammatory caspases such as caspase-1, -4, -5 and -11 are involved in inflammasome formation, as described further below [54].

Another factor tightly regulating apoptosis is the apoptotic-inhibitory molecule c-FLIP which counteracts caspase activation, and especially caspase-8 [55, 56]. c-FLIP can form heterodimers with procaspase-8 limiting the enzyme activity of caspase-8 (Figure 1.4) [57]. c-FLIP also contains DEDs which show similarity to caspase-8 in its death domains and therefore competes with binding to FADD [55, 58]. Death receptor-mediated apoptosis does not occur when c-FLIP is bound to FADD instead of caspase-8, as c-FLIP blocks this signaling [57, 59]. The *CFLAR* gene encodes three splice variants of c-FLIP [60]. These isoforms include c-FLIP Long (L), c-FLIP Short (S) and c-FLIP Raji (R) [55, 56]. All are found in humans, while c-FLIP (S) is not present in mice but c-FLIP (R) in mice is the functional counterpart to c-FLIP (S) in humans [55, 60]. The three isoforms perform different functions. The most studied isoform, c-FLIP (L), is a 55-kDa protein which is catalytically inactive [61]. It acts differently depending on its amount in the cell. In case of low amounts it works in a pro-apoptotic manner, while it acts in an anti-apoptotic way when the protein level is high [55]. c-FLIP (S) is a 26-kDa caspase-8 analog, and at high levels it works in an anti-apoptotic manner as it binds to and inhibit procaspase-8 to become its active form [55, 60]. This blocks death receptor-induced apoptosis [57]. The 24-kDa protein c-FLIP (R) is similar in structure as the short isoform [55, 60].

All three isoforms create heterodimers with procaspase-8 when recruited to the DISC and the cell fate is dependent on the relative amounts of (S) and (L) isoforms recruited to this complex [62]. However, only the c-FLIP (L)/procaspase-8 heterodimer performs catalytic activity and cleaves adjacent homo- and heterodimers. This generates the p43-c-FLIP (L) and p41/p43-procaspase-8 cleavage products [61]. The processing of p43-c-FLIP (L) can activate NF-κB signaling pathway and thereby act as an anti-apoptotic manner feedback mechanism [59]. The formation of the heterodimer inhibits procaspase-8 activation which makes RIPK1 form a complex with RIPK3 resulting in necroptosis. However, cleavage of RIPK1 by caspase-8 blocks necroptosis [63, 64].

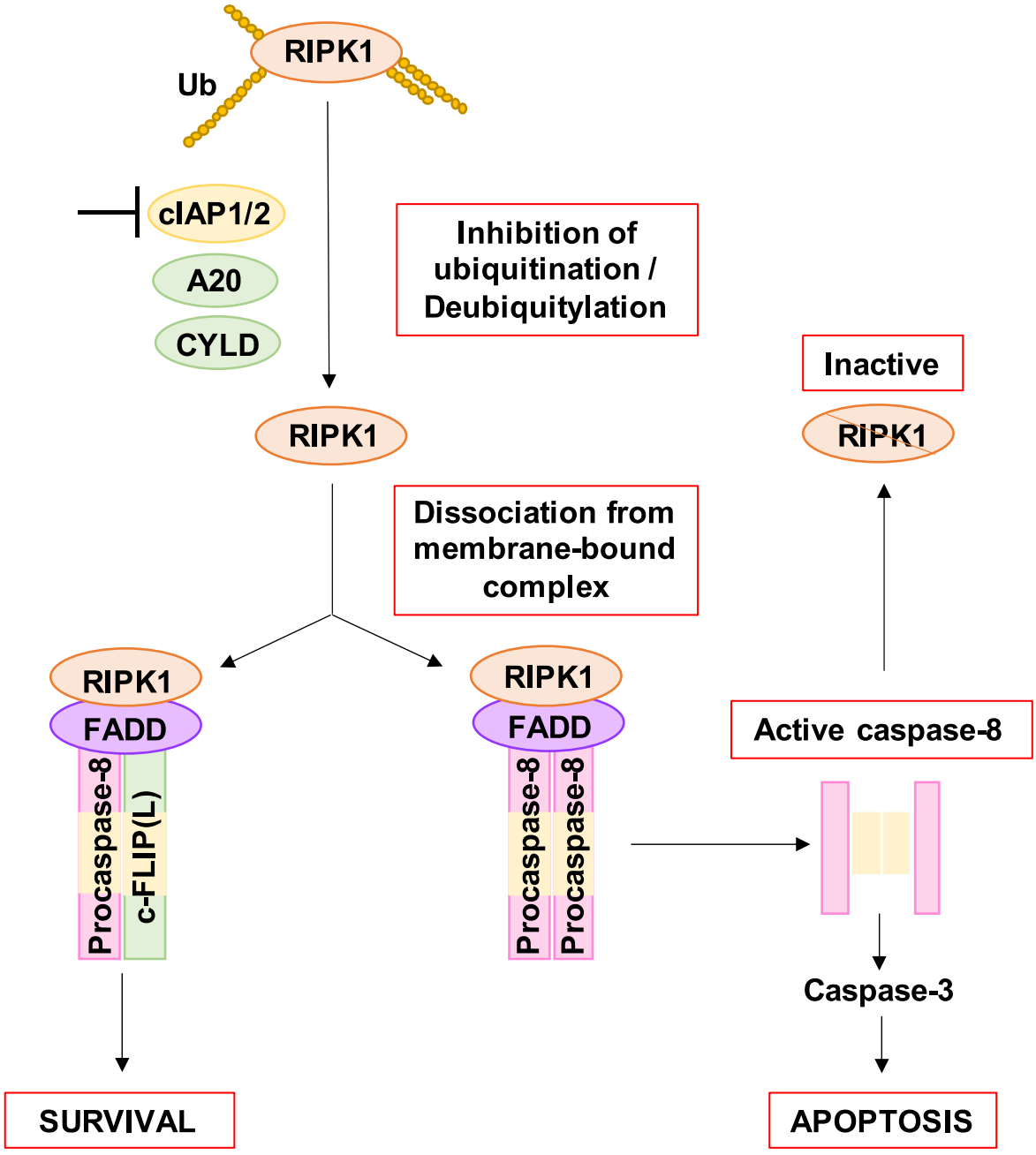


Figure 1.4 RIPK1-dependent apoptosis. Deubiquitylation of RIPK1 can be induced by deubiquinating enzymes (DUBs) such as A20 or CYLD, or by inhibiting ubiquitin ligases such as cIAPs or TRAFs. This results in dissociation of RIPK1 from the membrane-bound complex. In the cytosol, RIPK1 interacts with FADD which recruits procaspase-8 proteins forming homodimers. Active caspase-8 is induced by self-cleavage resulting in activation of caspase-3 and execution of apoptosis. Caspase-8 activity also inactivates RIPK1 by cleavage making it inaccessible to execute necroptosis. Alternatively, procaspase-8 can form heterodimers with c-FLIP (L) blocking caspase-8 activity inducing cell survival. Modified from [31].

1.5.2 RIPK1-dependent necroptosis

Necroptosis is a regulated lytic form of necrotic cell death causing inflammation due to the release of cellular content [65]. Cells undergoing necroptosis are characterized by loss of membrane integrity, they possess swelling organelles and leak their cellular content [66]. The cells emit damage-associated molecular patterns (DAMPs), such as adenosine triphosphate (ATP) and high mobility group box 1 (HMGB1), which induce the inflammatory responses [65, 67]. PRRs, such as TLRs, and TNF receptor superfamily recognize DAMPs to initiate an innate immune response [65, 68].

LPS stimulation of TLR4 or TNF-α binding to TNFR1 actives the receptor complex previously described, including RIPK1. Deubiquitylation of RIPK1 results in dissociation of RIPK1 from the membrane-bound complex. Active caspase-8 cleaves RIPK1 which makes it unavailable to procced to necroptosis [65, 69]. Inhibition of caspase-8 by for examples c-FLIP result in kinase activity of RIPK1 which is now able to interact with RIPK3 via their RIP homotypic interaction motifs (RHIMs). This results in phosphorylation and activation of RIPK3 by RIPK1 (Figure 1.5) [63, 70, 71]. Activation of RIPK3 leads to phosphorylation of the mixed lineage kinase domain-like protein (MLKL). Together, RIPK1, RIPK3 and MLKL form the necrosome [67, 72]. MLKL trimerizes upon phosphorylation by RIPK3 and interacts with the plasma membrane lipids and disrupt the integrity of the membrane to execute necroptosis. As a result, the membrane punctures and an influx of ions, such as calcium, occurs [65, 72, 73].

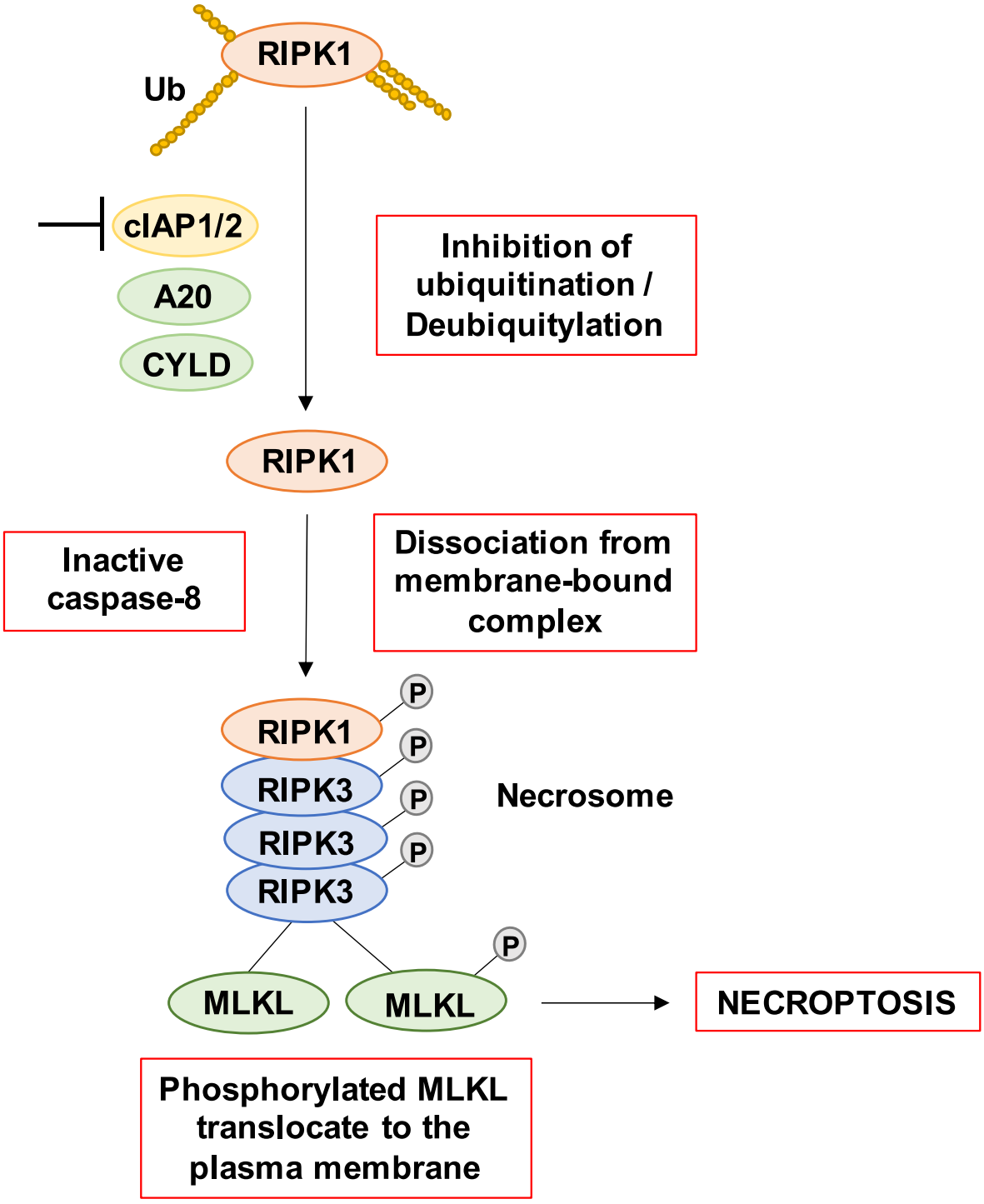


Figure 1.5 RIPK1-dependent necroptosis. Deubiquitylation or inhibition of ubiquitination proteins result in dissociation of RIPK1 from the membrane-bound complex when caspase-8 is inactive or blocked. Formation of the necrosome occur in the cytosol due to kinase activity of RIPK1 and RIPK3. RIPK1 recruits several RIPK3 proteins by trans- and autophosphorylation. Activation of MLKL occurs through phosphorylation and translocates to the plasma membrane resulting in induction of necroptosis. Modified from [31, 70].

1.5.3 Pyroptosis and the inflammasome

Pyroptosis is another mode of inflammatory cell death and shares a resemblance with necroptosis as it leads to lysis of the cell and inflammation. A pyroptotic cell exhibits bubbles on the plasma membrane and swelling features [74]. This type of cell death induces mechanisms in the innate immune system when intracellular pathogens are exposed to the cell. In contrast to necroptosis, pyroptosis is caspase-dependent and is activated through a multimeric protein complex, called the inflammasome. Induction of the inflammasome occurs when the cell is exposed to infection or stress [51].

Several types of inflammasomes exist and are provoked by different mechanisms [75]. Nucleotide-binding leucine-rich repeat receptors (NLRs) or absent in melanoma 2 (AIM2)-like receptors (ALRs) initiate the assembly of the inflammasome complex. NLR family pyrin domain containing 3 (NLRP3) is the best characterized NLR and is present in the cytosol [76, 77]. Two signals are needed for canonical NLRP3 inflammasome activation in macrophages: priming and activation. Priming of PRRs including TLR4 by inflammatory stimuli such as LPS prime the receptor to induce NLRP3 and pro-IL-1β expression. DAMPs and PAMPs activate NLRP3 which recruits apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC). ASC recruits procaspase-1 to form the canonical NRLP3 inflammasome which initiate procaspase-1 self-cleavage to become the active caspase-1. During necroptosis, membrane permeabilization also activates the NLRP3 inflammasome [76, 77, 78].

Caspase-1 is responsible for converting pro-IL-1β to IL-1β by proteolytic processing. The cell secretes IL-1β when the cell undergoes pyroptosis [76, 77, 78]. Caspase-1 also cleaves Gasdermin D (GSDMD) into a pore-forming protein. N-terminal GSDMDs (p30) translocate and permeabilize the plasma membrane by binding to phospholipids in the membrane and forming pores. The pores cause rupture in the cell membrane leading to IL-1β release and efflux of potassium ions, which induce pyroptosis [77, 79, 80].

Caspase-8 is mainly an apoptotic factor but is also involved in pyroptosis as an inflammasome-caspase. It can cleave pro-IL-1β and GSDMD during extrinsic apoptosis (Figure 1.6). This may induce pyroptosis [51, 76]. Cleavage of pro-IL-1β result in mature IL-1β which is released by the cell when pyroptosis is induced [77, 81]. Caspase-8 can also directly process and cleave

procaspase-1 and procaspase-3 to become the active caspases [82]. Activation of caspase-3 results in cleavage of GSDMD p43 but inhibits the N-terminal GSDMD which consequently blocks pyroptosis [83].

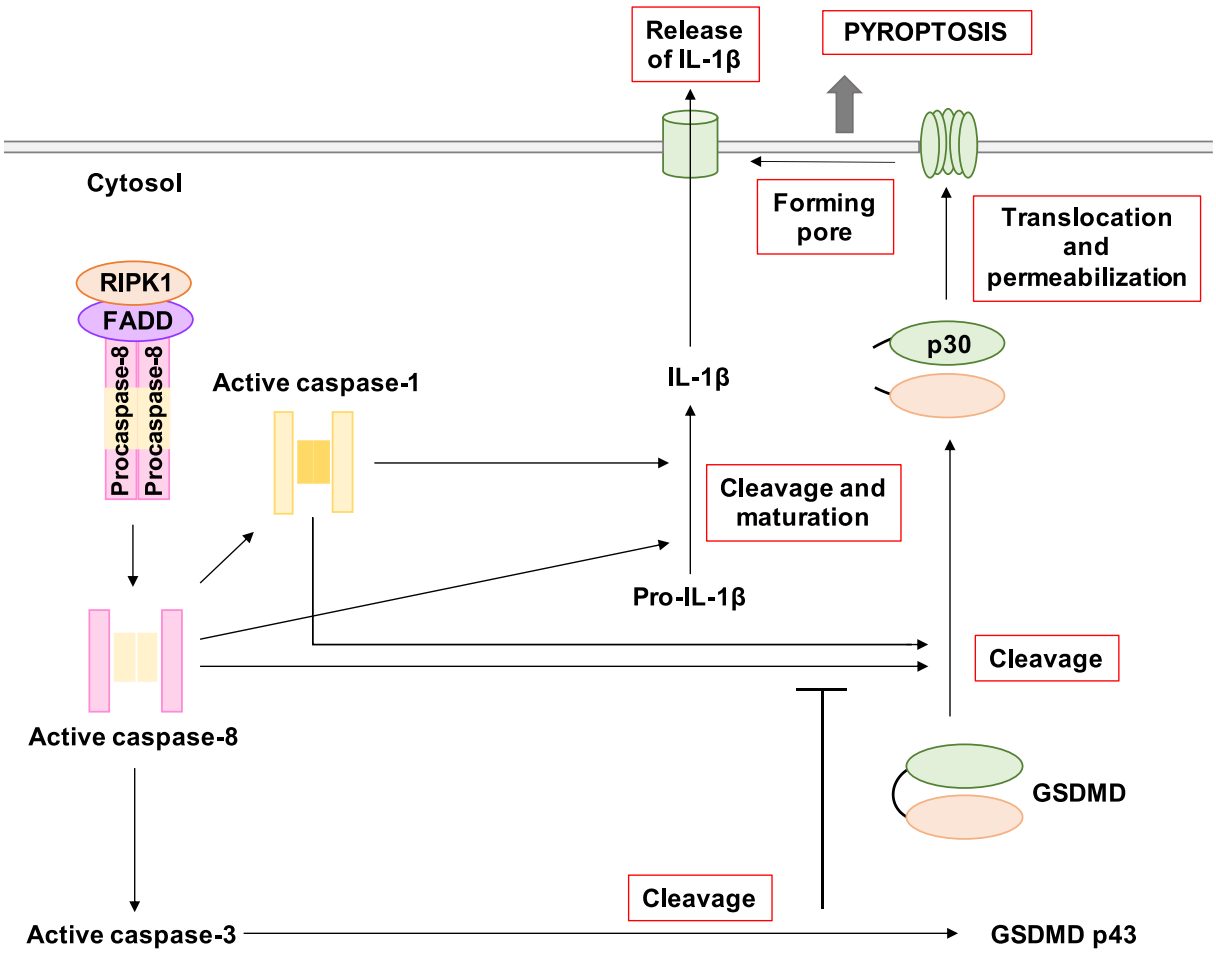


Figure 1.6 Extrinsic apoptosis induces caspase-8-mediated pyroptosis. Activation of caspase-8 via extrinsic apoptosis activates caspase-3 and in certain cases caspase-1. Caspase-8 cleaves GSDMD and pro-IL-1β directly or indirectly via caspase-1. Cleaved GSDMDs p30 translocate to the plasma membrane forming pores and inducing pyroptosis. Additionally, cleavage and maturation of pro-IL-1β to IL-1β by the caspases are released through the GSDMD-mediated pores. Activation of caspase-3 results in cleavage of GSDMD p43 and inhibits cleavage of GSDMD to p30 which leads to blocking of pyroptosis. Modified from [74, 83].

1.6 Targeting RIPK1 signaling

RIPK1 is essential in the human innate immune system as it mediates several signaling pathways involved in the immune response [30]. Dysregulation or mutation of RIPK1 or other factors in the RIPK1 pathway cause inflammation due to overproduction of inflammatory cytokines or uncontrolled cell death [84, 85]. Cytokine overexpression can result in disease development, such as inflammatory bowel disease (IBD), polyarthritis, rheumatoid arthritis (RA), multiple sclerosis (MS), and other autoinflammatory diseases [30, 33, 86]. Functional RIPK1 is crucial for triggering a host defense against pathogens [54]. Mutations of the *RIPK1* gene cause recurrent viral, bacterial, and fungal infections in humans, while it is lethal in mice [30, 33, 86]. RIPK1 inhibitors are desired for treating human inflammatory diseases. Necrostatins are small molecules that hamper necroptosis by inhibiting the kinase activity of RIPK1. The prevention of RIPK1-mediated necroptosis can be a potential therapy in sepsis, and neurodegenerative and autoimmune disorders [33, 86]. Since RIPK1 controls a broad spectrum of pathways, it is a desired target for medical drugs [30, 33].

1.6.1 IAP antagonists

The IAP proteins regulate pro-inflammation and PANoptosis by ubiquitinating and deubiquitylating RIPK1, respectively [87]. As cIAP1/2 are involved in apoptotic evasion in tumor cells, much attention has been directed at developing IAP antagonists, also termed second mitochondrial activator of caspases (SMAC)-mimetics (SMs). As cIAP1/2 are important in inflammation, they have also been explored as treatment for inflammatory diseases. SMs are small molecules inhibiting IAPs [27, 88, 89]. The IAP proteins possess IAP-Binding Motifs (IBMs) [39]. The antagonists mimic the minimal N-terminal tetrapeptide (NH_2-AVPI) SMAC, which is part of the IBM, and SMs can therefore bind to the BIR2 and BIR3 domains of cIAP1/2. The binding results in inhibition of the cIAPs, which no longer can ubiquitinate RIPK1 resulting in apoptosis or necroptosis [39, 47]. Apoptosis is the primary mode when SMs induce cells to undergo cell death, but cells possessing the necroptotic machinery can switch to this cell death by inhibiting caspase-8, or if caspase-8 levels are low [44]. Reduction in ubiquitination of RIPK1 by SMs inhibiting cIAP1 and cIAP2 induce expression of survival genes, such as *CFLAR* encoding the protein c-FLIP [47]. Monovalent SMs have one AVPI-like binding motif, while bivalent SMs present two such motifs [39].

Birinapant, a second-generation bivalent IAP antagonist, can induce cIAP1 and cIAP2 degradation. The SM seems to have a higher affinity for cIAP1 than cIAP2 [36, 39, 41]. Activation of NF-κB through TNF-α-signaling is inhibited by birinapant, resulting in cell death [36].

LCL-161, a monovalent SM, is another IAP antagonist. The antagonist stimulates the degradation of cIAP1 [39]. LCL-161 inhibits IAPs in different cell types, and it seems to induce IL-1β production in a slightly greater extent than birinapant in different models [47]. In addition, LCL-161 treatment down-regulate the expression of the anti-apoptotic protein c-FLIP in pro-inflammatory macrophages [90].

IAP antagonists are the most common clinical approach to induce RIPK1-dependent cell death by activating the DISC. As single agents, the SMs exhibit small effects but have increased efficiency in combination with other therapies, such as immunotherapy, kinase inhibitors, radiotherapy, and chemotherapy [44]. Not all patients respond to a single IAP antagonist alone but can have some effect combined with another SM. Due to the high specificity of the target, the SMs are safe to use in humans [39]. However, IAP antagonists are responsible for causing Bell's palsy in some patients [89]. Bivalent SMs, such as birinapant, seem to have a higher binding affinity to the IAP than the monovalent IAP antagonists, including LCL-161 [89].

1.6.2 Pro-inflammatory human macrophages as a therapeutic target for SMs

Pro-inflammatory human macrophages are involved in chronic autoimmune and inflammatory diseases, including RA, atherosclerosis and MS [90]. These diseases cause inflammation in affected parts of the body due to dysregulation of the immune system [27]. In RA for instance, macrophages play an essential role as they produce cytokines that increase the inflammation by recruiting additional immune cells. In addition, the cytokines produced by the macrophages contribute to destruction of bone and cartilage. Progression of this disease is also mediated by macrophages as they produce reactive oxygen species (ROS), nitric oxide intermediates and matrix-degrading enzymes [91]. The symptoms of inflammatory diseases can be alleviated by inducing cell death in overactive macrophages with the use of SMs [27]. Monocytes and pro-inflammatory macrophages are known to be highly susceptible to SM-induced cell death and can therefore be a potential target to chronic autoimmune and inflammatory diseases [90]. However, due to the diverse function of IAPs in inflammation and cell death signaling it is important to understand these pathways in order to prevent unwanted effects [27].

1.7 Aim of study

Previous work in our laboratory found that LPS-pretreated pro-inflammatory macrophages were more susceptible for birinapant-induced cell death, as compared to CSF-1 and anti-inflammatory macrophages, and that this death was accompanied by IL-1β production. Preliminary experiments suggested that the mode of death might be apoptosis. In this work we aimed at further characterizing this process.

Mice have been the common model organism for studying IAP inhibitors, and studies testing SMs in human macrophage systems are lacking. It is necessary to investigate the role of SMs in human cells in order to safely introduce the inhibitors as therapeutic drugs. Therefore, we used human macrophages in our experiments to investigate our aim.

The aim was divided into the following objectives:

- Investigate if time of LPS pretreatment affected birinapant response
- Investigate whether LPS pretreatment also sensitized macrophages to the SM LCL-161
- Test whether TNF-α pretreatment potentiated birinapant-induced cell death
- Investigate whether c-FLIP levels were influenced by LPS and TNF-α
- Evaluate the mode of cell death induced by assessing apoptotic-, necroptotic- and pyroptotic-related proteins

2 Materials and methods

2.1 Cells and culture conditions

All experiments in this thesis were performed on macrophages differentiated from primary human monocytes. The monocytes were isolated from one-day-old buffy coats from the Blood bank at St. Olav's hospital. All donors were healthy individuals with either blood type A, Rh-positive or blood type B, Rh-positive.

Monocytes were isolated from peripheral blood mononuclear cells (PBMCs). The blood was mixed with Dulbecco Phosphate buffered saline (DPBS, Sigma-Aldrich, #D8537) and distributed into 50 mL tubes. To obtain the PBMC layer, density centrifugation was performed with Lymphoprep^{TM} (Alere Technologies AS, #07851) at 645 x g, 20ºC for 20 minutes. Erythrocytes and granulocytes sedimented through the Lymphoprep^{TM}, while the PBMC layer were retained in the interface between the plasma and the Lymphoprep^{TM}.

The PBMC layer was transferred to new 50 mL tubes. To remove serum and Lymphoprep, the PBMC was centrifuged (796 x g, 10 min, 20 ºC) in Hanks' balanced salt solution (Sigma-Aldrich, #H9269), and the supernatant was carefully removed. The pellet was resuspended in preheated Hanks and centrifuged at 127 x g, 20 ºC for 8 minutes. This step was repeated three times. The number of cells was counted using Cell Counter (Beckman Coulter Inc.) in isotone. ZAP-OGLOBIN II Lytic Reagent (Beckman Coulter Inc., #7546138) was added to lyse the red blood cells during counting.

Monocytes were isolated from the PBMCs using magnetic beads which bind to $CD14^+$ monocytes (MACS Miltenyi Biotec, #130-118-906). An LS column (MACS Miltenyi Biotec, #130-042-401) was placed on a magnetic field and rinsed with sterile filtered MACS wash-buffer (auto MACS Rinsing Solution, #130-091-222) with 1:20 BSA Stock Solution (MACS Miltenyi Biotec, #130-091-376). The PBMCs was centrifuged at 300 x g for 10 minutes at 20ºC, and the supernatant was aspirated completely. The pellet was resuspended in MACS wash-buffer before $CD14^+$ magnetic beads were added and incubated for 15 minutes at 4ºC. 10^8 cells were used per column. The cells were resuspended in MACS wash-buffer and centrifuged at 300 x g for 10 minutes at 20ºC. The supernatant was aspirated completely, and the pellet was resuspended in MACS wash-buffer. The cell suspension was applied to the LS column. $CD14^+$ cells were binding to the magnetic beads, which were retained in the column matrix due to a magnetic field. The rest of the cell suspension passed through the column, and the column was

washed three times with MACS wash-buffer. The column was removed from the magnetic field, and the magnetically labeled $CD14^+$ cells were flushed into a new clean tube using wash-buffer and the accompanying plunger. The Cell Counter was used to determine the number of cells before they were seeded out.

To be differentiated into macrophages, the isolated monocytes were cultured in RPMI (RPMI-1640 Medium, Sigma-Aldrich, #R8758) with 20% fetal calf serum (FCS) and CSF-1 (100 ng/mL). The cells were cultured at 37ºC with 5% CO_2 for 7 days. Then, the macrophages were treated with LPS (50 ng/mL) or TNF-α (1 ng/mL). The medium was changed to Opti-MEM reduced serum medium (Gibco® - Life technologies, #11058021) before further treatment of the cells. All stimuli and treatments used in the experimental setups in this thesis are included in Table 1.

Table 1 Treatments and stimuli used in experimental setups.

Compound	Property	Concentration	Manufacturer (Cat.No)
Birinapant	cIAP1- and cIAP2-inhibitor	1-6 µM	Selleckchem (#S7015)
CSF-1	Differentiates monocytes to macrophages, cytokine	100 ng/mL	R&D Systems (#216-MC-010)
LCL-161	XIAP- and cIAP1-inhibitor	1 µM	Selleckchem (#S7009)
LPS	Gram-negative lipopolysaccharide, TLR4 ligand	50 ng/mL	InvivoGen (#tlrl-3pelps)
TNF-α	Pro-inflammatory cytokine, TNFR1 ligand	1 ng/mL	PeproTech Nordic (#300-01A)
zVAD-fmk	Pan-caspase-inhibitor	20 µM	R&D Systems (#FMK001)

2.2 Cell cytotoxicity and viability

2.2.1 LDH Cytotoxicity Detection kit

Lactate dehydrogenase (LDH) Cytotoxicity Detection kit (Takara Bio, #MK401) was used to measure cell death. The cytosolic enzyme LDH is present in all cells and is released into the supernatant during cell death. The kit is a colorimetric assay measuring LDH as an estimate of cellular cytotoxicity. In a two-step enzymatic reaction, the extracellular LDH first catalyzes the conversion of lactate to pyruvate by reducing NAD^+ to $NADH/H^+$. In the second step,

$NADH/H^+$ is then used by diaphorase to reduce a tetrazolium salt (INT) to red formazan product. The amount of released LDH by dead cells is directly proportional to the amount of formazan dye measured in the samples [92].

After 18 hours of treatment, the supernatant from the cells was transferred to a 96-well plate containing the reaction mixture. This mixture consisted of the dye solution (INT/Na-lactate) and the catalyst (diaphorase/NAD^+, 1:45). The plate was incubated for 15-20 minutes in the dark at room temperature. Then, the absorbance was read at 490 nm using the iMark Microplate Absorbance Reader (Bio-Rad, #11843) and the Microplate Manager® Software.

The positive control for all experiments were cells lysed by adding Triton X-100 (1%, Sigma-Aldrich, #T8787) to the cell culture medium 20 minutes before collecting supernatant. The samples treated with the inhibitor solvent, Dimethyl sulfoxide (DMSO, Sigma-Aldrich, #D2650), were used as negative control for all experiments. When calculating the cell death, the positive control was set as 100% cell death, while the negative control was set as 0% cell death.

2.2.2 Cell Titer Proliferation Assay

Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, #G3580) was used to measure cell viability in the experiments. It is a colorimetric assay containing a tetrazolium compound (MTS) and the electron coupling reagent phenazine ethosulfate (PES). MTS uses NADH/NADPH to convert to a colored formazan product, which is produced in metabolically active cells by dehydrogenase enzymes. The amount of soluble formazan product in the cell culture medium was measured and is directly proportional to the number of living cells. The measurement of formazan product was performed at 490 nm absorbance.

After stimulation for 18 hours, the reagent was added to the wells containing cultured tissue media and incubated in the dark (37ºC, 5% CO_2, 2 hours). The measurement was performed at 490 nm absorbance with the iMark Microplate Absorbance Reader (Bio-Rad, #11843) and the Microplate Manager® Software. For all experiments, the negative control was cells lysed by 1% of Triton-X 100 (treated 20 minutes prior to adding solution reagent) and was set as 0% viability. The positive control was samples treated with DMSO, illustrating 100% viable cells.

When calculating the percentage of viable cells, the negative control was set to 0% viability and the positive control as 100%.

2.3 Sandwich ELISA

Enzyme-linked immunosorbent assay (ELISA) is a technique that utilizes antibodies for detecting and quantifying specific soluble targets such as cytokines and proteins in a liquid sample. In a sandwich ELISA, the multi-well plate surface is coated with capture antibodies that capture the target antigen in the solution. Then, the detection antibody detects the target before binding to horseradish peroxidase (HRP) conjugated with streptavidin. This forms a "sandwich" containing antibody-antigen-antibody, giving a double-layer of specificity. By using a spectrophotometer, the substrate oxidation can be detected by measuring the absorbance.

To detect IL-1β in the supernatant of our samples DuoSet® ELISA Development system was used (R&D systems, #DY201). The Capture Antibody was diluted to a 4 µg/mL working concentration in PBS and used to coat a 96-well microplate in the first preparation step. The plate was sealed and incubated overnight at room temperature. Then, Tween (0.05%) in PBS was used to aspire and wash the microplate three times using the 96 PW-Tecan plate washer (Tecan, Bergman Diagnostics, #16029011). The microplate was inverted and blotted against paper towels to completely discard the remaining wash-buffer in the wells from the last wash. The blocking step was performed by adding Reagent Diluent (1% BSA in PBS) to the microplate before incubated at room temperature for 1 hour. Then, the washing-procedure was repeated. The samples and standards were diluted in Reagent Diluent and added to the microplate before it was sealed and incubated at room temperature for 2 hours. The washing-procedure was repeated. The Detection Antibody was diluted in Reagent Diluent (150 ng/mL) and added to the microplate before it was sealed and incubated at room temperature (2 hours). The plate was again aspirated three times prior to the addition of Streptavidin-HRP diluted in Reagent Diluent, and the microplate was sealed and incubated in the dark for 20 minutes at room temperature. The washing-procedure was repeated as earlier before Substrate solution was added (1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine, TMB), Biolegend, #421101) and incubated in the dark at room temperature (20 minutes). When peroxidase (H_2O_2) catalyzes TMB, a pale blue color is produced. For the final step, stop solution (2N H_2SO_4, VWR Chemicals, #20I044003) was

added to stop the reaction. Stop solution consists of sulfuric acid that inhibit the blue oxidation product of TMB to develop and converts it to a yellow derivative. The optical density was determined immediately after addition of stop solution by using iMark Microplate Absorbances Reader (Bio-Rad, #11843) at 450 nm and Microplate Manager® Software.

2.4 Cell lysis, Gel electrophoresis and Immunoblotting

To analyze proteins by gel electrophoresis and immunoblotting, the following procedure was performed. The cell culture was put directly on ice. The medium was removed from the cell culture, and ice-cold PBS was used to wash the samples. The PBS was removed, and the cells were then lysed directly on the cell culture plate. Ice-cold lysis buffer containing Tris-HCl (50 nM), NaCl (150 nM), NP-40 (1%), Ethylenediaminetetraacetic acid (EDTA, 1 mM) and glycerol (10%) was added to all samples. Right before use, the protease inhibitor (Complete Mini EDTA-free, Roche Life Science, #11873580001) and the phosphatase inhibitors, sodium fluoride (NaF, 20 mM) and sodium orthovanadate (Na_3VO_4, 0.7 mM), were added to the lysis buffer. The samples were lysed for 15 minutes while stirring and shaking the dish with the cells before the suspension were transferred to Eppendorf tubes. The tubes were centrifuged (15,000 x g for 15 minutes at 4ºC) to remove DNA and other unwanted cell debris which formed a pellet. The supernatant containing the proteins were transferred to new Eppendorf tubes. Dithiothreitol (DTT, 1:10) and Sample Buffer (NuPage Invitrogen, #NP0007) were added to the lysate. DTT was added to reduce the disulfide bonds of the proteins. The Sample Buffer contains sodium dodecyl sulfate (SDS), which breaks and linearize the secondary and tertiary structures of the proteins. To denaturize the proteins before analysis, the samples were put on a heating block (70ºC) for 15 minutes. The proteins form coils where the negative charge and size are proportional to their mass. Then, the samples were stored at -80ºC.

Gel electrophoresis pre-cast gels with 4-12% polyacrylamide (Bis-Tris NuPage, Invitrogen, #NP0321BOX, #WG1402BX10) were used to separate the protein fragments by size. The pre-cast gel was placed in an XCell SureLock™ Mini-Cell or XCell4 SureLock™ Midi-Cell (NuPage, Life Technologies, #EI0001, #WR0100) before adding 1x MES SDS running buffer (20x, NuPage, Life Technologies, #NP0002). The samples were loaded to the gel with equal amounts of protein. SeeBlue™ Plus2 (Thermo Fisher Scientific, #LC5925) was loaded to the gel as a ladder before a three-step program was used to run the gel (step 1: 80V – 30 minutes, step 2: 150V – 30 minutes, step 3: 180V – 30 minutes).

The iBlot™ 2 Dry Blotting System (Invitrogen, Thermo Fisher Scientific, #IB21001) was used according to manufacturer's instruction to blot the gel containing the separated proteins from the gel electrophoresis. The blotting followed a three-step program (step 1: 20V – 1 minute, step 2: 23V – 4 minutes, step 3: 25V – 2 minutes). Then, the membrane was removed and rinsed for 5 minutes with Tris-buffered saline (Oxoid, #BR00140) with 0.1% Tween-20 (TBS-T) (Sigma-Aldrich, #P1379) on a roller plate. Bovine Serum Albumin (BSA, 5%) in TBS-T was used for 1 hour to block the membrane to prevent unspecific binding of antibodies. Then, the membrane was incubated with primary antibodies (Table 2) in 1% BSA at 4ºC. Incubation time varied between antibodies. TBS-T was used to wash the membrane three times (5 minutes each) prior to the addition of secondary antibodies (Table 3) at room temperature (1 hour). Either fluorescence-conjugated or HRP-conjugated antibodies were used. The membrane was rinsed in TBS-T three times for 5 minutes before developing.

Odyssey® CLx Imaging System (Li-Cor) and Image Studio™ Software (Image studio, Ver 5.2, Li-Cor) were used to develop the membranes with fluorescence-conjugated secondary antibodies. Prior to detecting of the polyclonal Goat Anti-Rabbit or Anti-Mouse IgG/HRP probed membranes, SuperSignal (Thermo Fisher Scientific, 1:1, 2 mL, #34096) was added to the membranes (2 minutes). SuperSignal is an enhanced chemiluminescent (ECL) substrate that is ultra-sensitive and binds to the enzyme horseradish peroxidase (HRP), which is conjugated to the secondary antibodies used. Odyssey® Fc Imaging System (Li-Cor Biosciences) and the Image Studio™ Software were used to analyze the membranes.

Table 2 Antigens and primary antibodies used for Western Blot staining.

Antigen, source species for antibody	**Expected molecular weight (kDa)**	**Manufacturer, Cat.No**
GAPDH, mouse IgG1	36	Abcam, ab8245
β-tubulin, rabbit IgG	55	Abcam, ab6046
Caspase-3, rabbit IgG	17, 19, 35	Cell Signaling Technology, 9662
Caspase-8, mouse IgG	18, 43, 55	Enzo, ALX-804-242-C100
GSDMD, rabbit IgG	21, 30, 43, 53	Cell Signaling Technology, 97558
FLIP, rabbit IgG	25, 43, 55	Cell Signaling Technology, 56343
phospho-RIPK1(Ser166), rabbit IgG	78 – 82	Cell Signaling Technology, 44590
phospho-RIPK3(Ser227), rabbit IgG	46 – 62	Cell Signaling Technology, 93654

Table 3 Secondary antibodies used for Western Blot staining.

Antibody	**Manufacturer, cat.no**
Polyclonal Goat anti-Mouse IgG, 680 RD	Li-Cor, 926-68070
Polyclonal Goat anti-Rabbit IgG, 680 RD	Li-Cor, 926-68071
Polyclonal Goat anti-Mouse IgG/HRP	Dako Denmark A/S, P0447
Polyclonal Goat anti-Rabbit IgG/HRP	Dako Denmark A/S, P0448

2.5 Statistics

All experiments were performed on multiple donors. For multiwell-experiments the experiments were performed in technical triplicates for each donor and the average of technical triplicates from each donor was used as input when comparing between donors. The number of donors for each experiment is specified in the figure texts. Experiments are presented with all donors (multiwell experiments) or with one representative donor (immunoblotting), and the statistical analysis is performed on all donors. The statistical tests performed were One-way ANOVA for comparison of multiple groups and Two-way ANOVA for an interaction-comparison between multiple groups split by two independent variables. Both ANOVAs were performed with a Bonferroni posttest. Prism software 5 (GraphPad Software Inc, La Jolla, CA, US) was used for both comparisons. Statistical significance is denoted in the figures with an asterisk, with $p < 0.05$ considered statistically significant. Data are given with error bars presenting standard deviation to show the variation around the mean.

3 Results

RIPK1 is a central regulator of cell death and inflammation in macrophages [31]. SMAC-mimetics (SMs) inhibit cIAPs which lead to deubiquitylation of RIPK1 and a shift in signaling from cell survival to death. The aim of this thesis was to test if the length of pretreatment by LPS or TNF-α potentiated the cytotoxicity of birinapant-induced cell death *in vitro* and to evaluate the mode of cell death induced.

3.1 The effect of LPS-pretreatment on SMAC-mimetic cytotoxicity

Earlier work in our group showed that LPS-pretreatment of macrophages for 18 hours sensitized the cells for SM-induced cell death (Klaharn 2019, *Unpublished*). Therefore, we wanted to study if this was the occurrence for shorter LPS-pretreatment. Human $CD14^+$ monocytes from five independent donors were isolated and differentiated to macrophages as described in the methods section. After 7 days the macrophages were pretreated with LPS in a time series before medium containing LPS was removed, cells washed, and then stimulated with birinapant or LCL-161 for 18 hours (Figure 3.1, A).

In the birinapant-experiments we also included a time-series of LPS alone to monitor whether LPS had any effect of cell death or viability by itself. As we did not see any effect, this was omitted for subsequent experiments.

To test the effect of cell death and viability after birinapant or LCL-161 treatment, two independent methods were performed on the same samples. The cell death was analyzed with LDH Cytotoxicity Detection Kit, and Cell Titer Proliferation Assay was used to determine cell viability (Figure 3.1).

Birinapant treatment on LPS-pretreated macrophages showed a reduction in cell viability for all time points compared to birinapant alone, although only pretreatment for 6 and 18 hours were statistically significant (Figure 3.1, C). A similar effect on cell death was observed (Figure 3.1, B) but this was not statistically significant. Our results also showed that birinapant alone has a significant effect on reducing cell viability of untreated macrophages (Figure 3.1, B).

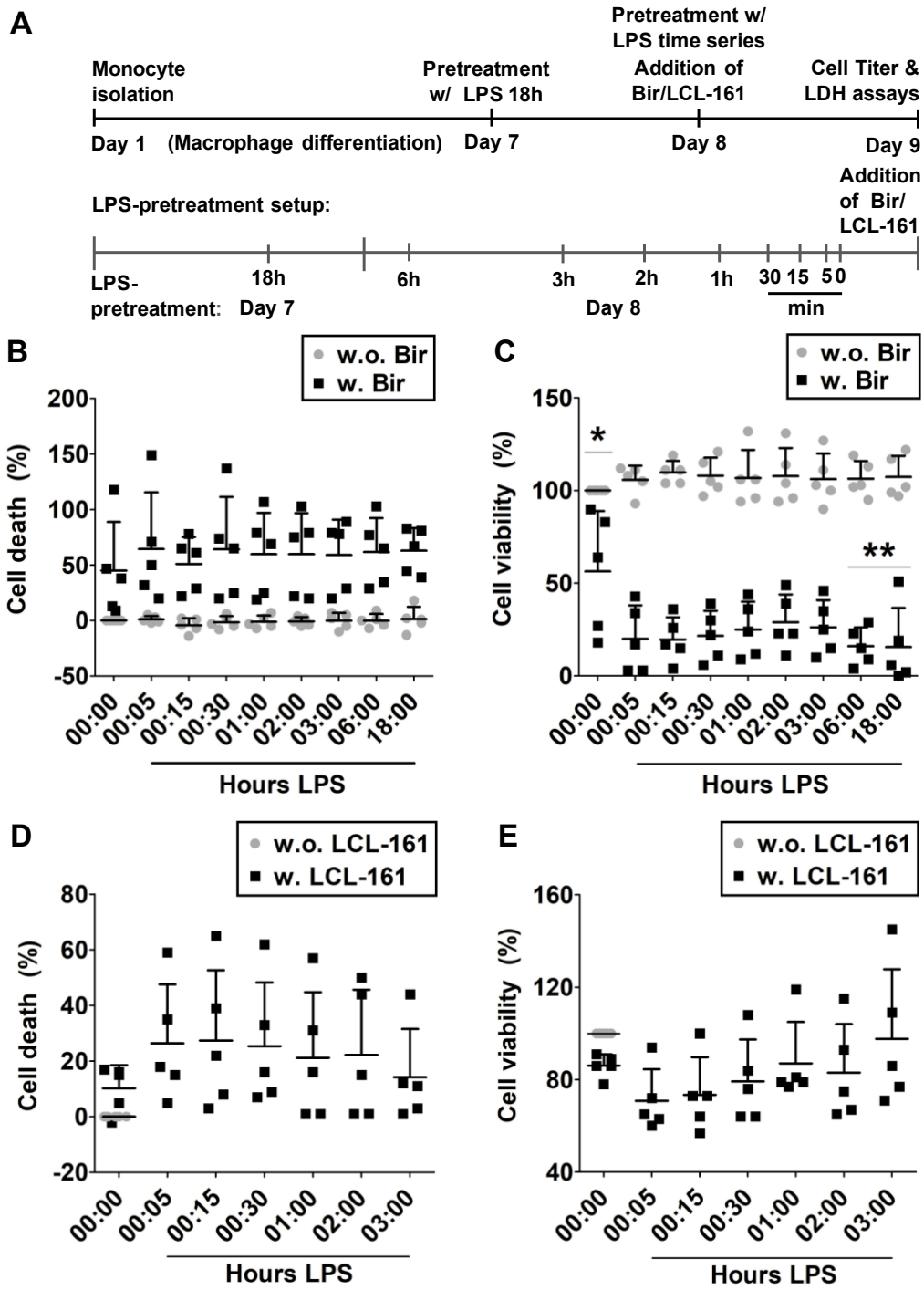


Figure 3.1 The effect of LPS-pretreatment on SM cytotoxicity. A, B, C, D, E. Timeline of macrophage differentiation and pretreatment setup (**A**). Human macrophages were pretreated with 50 ng/mL LPS for the indicated time, before cells were washed and treated with 1 µM birinapant (**B, C**) or 1 µM LCL-161 (**D, E**) in opti-MEM o/n (15-20 hours). 1 µM DMSO was used as control (**B**-**E**). The cell death was measured by the cytotoxicity of LDH-release and cell viability was measured by Cell Titer Proliferation Assay. All experiments were performed on at least five donors, and individual donors, average and standard deviation are indicated. Single asterisk denotes statistical significance between untreated and SM alone, while double asterisk denotes statistical significance between LPS+SM and the SM alone (**C**), ($p < 0.05$, *B - E: Two-way ANOVA and one-way ANOVA, with Bonferroni posttest).*

LCL-161 treatment induced a slight reduction in viability and a corresponding increase in cell death. LPS-pretreatment increased this effect (Figure 3.1, D, E). However, none of the effects were statistically significant.

The results indicated that there were donor variations in both experiments, but this was more prominent for observation with LCL-161 treatment. Both SMs showed some enhanced effect with LPS-pretreatment but due to the donor variation most of the results are not statistically significant.

Together, these experiments showed that stimulation with birinapant on LPS-pretreated macrophages reduced the cell viability in these cells for all time points, but a significant effect was first observed after 6 hours of pretreatment. The results also indicated that LCL-161 did not exhibit the same potency on cell death as birinapant on LPS-pretreated macrophages.

3.2 TNF-α-pretreatment does not potentiate birinapant-induced cytotoxicity in human macrophages

After having demonstrated that birinapant potentiated cytotoxicity in macrophages pretreated with LPS at different time points, we wanted to investigate if TNF-α-pretreated macrophages displayed the same reduction in cell viability when treated with this SM. The background for this experiment was that LPS and TNF-α pretreated macrophages both activate NF-κB through RIPK1 signaling [44].

Human macrophages were pretreated with TNF-α at different time points in a time series of 3 hours to test the effect of birinapant on five independent donors (Figure 3.2, A, B). The experimental setup of the cell death and viability analysis was performed similar as the experiments with LPS and LCL-161.

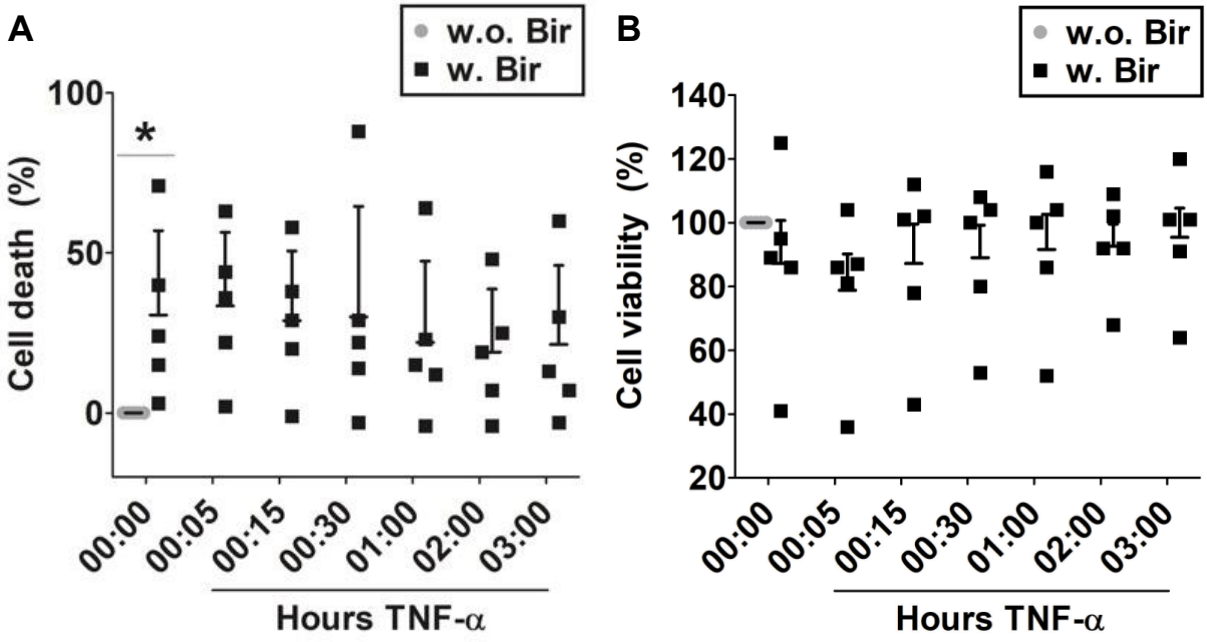


Figure 3.2 TNF-α-pretreatment does not potentiate birinapant-induced cytotoxicity in human macrophages. A, B. Human macrophages were pretreated with 1 ng/mL TNF-α for the indicated time, before cells were washed and treated with 1 µM birinapant in opti-MEM o/n (15-20 hours). 1 µM DMSO was used as control. The cell death was measured by the cytotoxicity of LDH-release (**A**) and cell viability was measured by Cell Titer Proliferation Assay (**B**). All experiments were performed on five donors, and individual donors, average and standard deviation are indicated. Asterisk denotes statistical significance between untreated and birinapant alone (**A**) ($p < 0.05$, *Two-way ANOVA and one-way ANOVA with Bonferroni posttest).*

Birinapant stimulation of TNF-α-pretreated macrophages showed a slight increase in cell death after 5 minutes of pretreatment compared to birinapant alone, although not significant (Figure 3.2, A). However, birinapant alone significantly increased cell death in untreated macrophages. The reduction in cell viability was modest for TNF-α-pretreated macrophages stimulated with birinapant and showed no significant effect (Figure 3.2, B). The cytotoxicity of birinapant treatment was not enhanced when stimulated on TNF-α-pretreated macrophages. The data also displayed great variance between donors.

Altogether, the cell death and viability experiments (Figure 3.1 and Figure 3.2) demonstrated that LPS-pretreatment, and not TNF-α-pretreatment, potentiated cytotoxicity in human macrophages treated with birinapant but the effect was first significant after 6 hours of pretreatment.

3.3 Birinapant induces IL-1β release in LPS-pretreated macrophages

Mature IL-1β is released from pyroptotic cells during lysis. Upon inflammasome activation GSDMDs translocate to the plasma membrane forming pores which also release IL-1β [74]. Preliminary studies performed by other members of our group have revealed that birinapant induced IL-1β production in macrophages after LPS-pretreatment for 18 hours (Klaharn 2019, *Unpublished*). We wanted to study the effect of birinapant on macrophages pretreated with LPS at different time points to test if also shorter pretreatment induced the same amount of IL-1β release. Supernatant obtained from the cell death/viability experiments were analyzed by sandwich ELISA to test the presence of IL-1β (Figure 3.3).

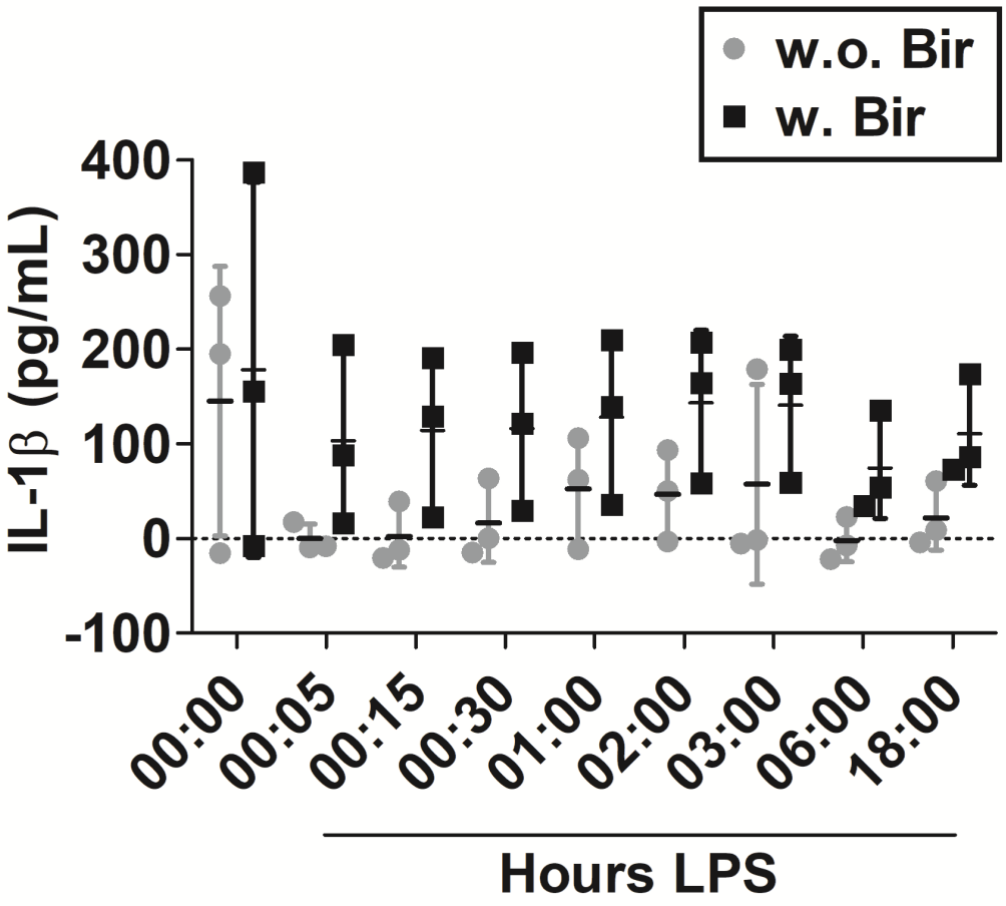


Figure 3.3 Birinapant induces IL-1β release in LPS-pretreated macrophages. Human macrophages were pretreated with 50 ng/mL LPS for the indicated time, before cells were washed and treated with 1 µM birinapant in Opti-MEM o/n (15-20 hours). 1 µM DMSO was used as control. The supernatant was analyzed for IL-1β by sandwich ELISA. All experiments were performed on three donors, and individual donors, average and standard deviation are indicated. The results showed no statistical significance between time points compared to the controls or comparisons between treatments within the time points *($p < 0.05$, Two-way ANOVA and one-way ANOVA with Bonferroni posttest).*

Analysis by sandwich ELISA showed that some untreated macrophages release IL-1β (Figure 3.3). This was independent of birinapant because both groups not pretreated with LPS were similar, and this is not consistent with what has been observed in the group previously. The results also indicated that the LPS-pretreatment of macrophages reduced this IL-1β release. However, birinapant treatment of LPS-pretreated macrophages induced IL-1β release. The birinapant-induced IL-1β release was not statistically significant, but this might be due to the low number of donors. As negative controls displayed high variation, we only included three donors in this experiment.

Within the same time point, birinapant stimulation on LPS-pretreated macrophages showed enhanced IL-1β release compared to its LPS control. However, most likely due to donor variation the results were not statistically significant.

3.4 c-FLIP protein levels increase in LPS-treated human macrophages

c-FLIP is a caspase 8-homologue that binds to caspase-8 and interferes with enzyme activity. It has been described as an anti-apoptotic protein blocking cell death [64]. The background for this experiment was based on earlier findings in our group which showed more c-FLIP in cells more prone to apoptosis (Moen & Klaharn 2019, *Unpublished*). In this thesis, the focus was to investigate in more detail how c-FLIP isoforms are influenced by LPS and TNF-α. Macrophages were treated with LPS or TNF-α in a time series, lysed and immunoblotted (Figure 3.4, A).

The immunoblotting and quantification showed a clear trend of increased levels of the c-FLIP isoforms Long (L), Short (S) and Raji (R) peaking at 6 hours with LPS-treatment. The increased levels of c-FLIP were significant at 6 hours for all three isoforms compared to untreated control (Figure 3.4, B, E, F). The protein levels of the (S) isoform had also a significant increase at 3 hours with LPS-treatment (Figure 3.4, E).

c-FLIP(L) is cleaved by the c-FLIP(L)/procaspase-8 heterodimer to c-FLIP(L) p43 [61]. Immunoblotting showed the presence of a double band at around 43 kDa (from now referred to as p43 high and p43 low) (Figure 3.4, A). The results displayed an oscillating trend for these proteins and a shift in levels between p43 high and p43 low during the time series. Quantification analysis showed that only p43 high had a significant effect of increased protein levels compared to the untreated macrophages (Figure 3.4, C). This significant effect was identified after 6 hours of LPS-treatment which is similar to the three isoforms.

Treatment with TNF-α had no significant effect on the c-FLIP levels compared to untreated. However, the results showed some increase of the p43 high protein levels from 3 hours to 18 hours (Figure 3.4, C). Altogether, the results showed a clear difference in c-FLIP levels after LPS-treatment (Figure 3.4).

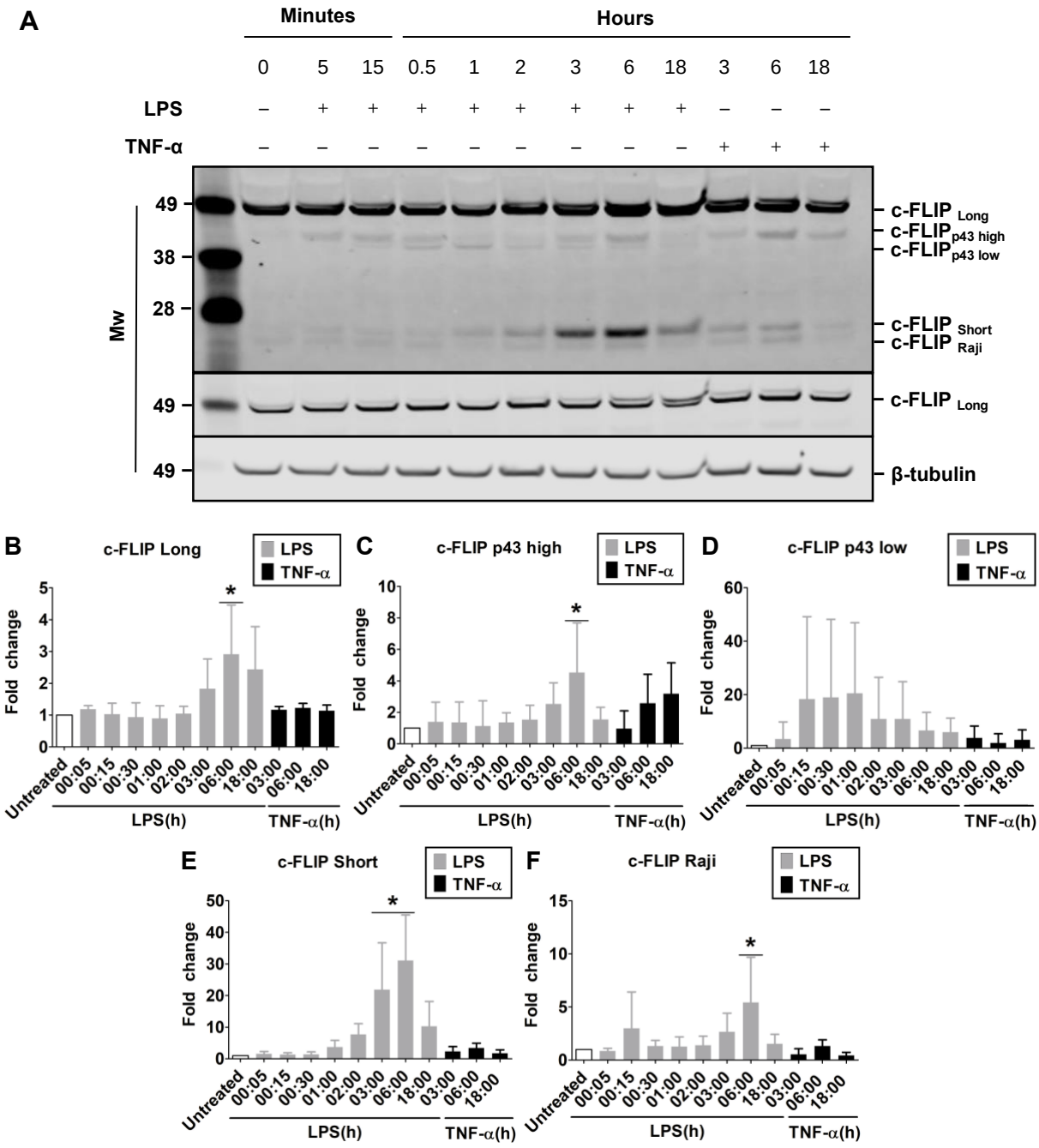


Figure 3.4 LPS increases protein levels of c-FLIP isoforms and p43 high cleavage product. A, B, C, D, E, F. Human macrophages were treated with 50 ng/mL LPS or 1 ng/mL TNF-α at indicated time points. Cell lysates were analyzed for c-FLIP isoform proteins and cleaved products by immunoblotting. One representative of five donors is shown. Immunoblots of all donors are found in Supplementary Figure S1. Additional image of c-FLIP Long is from the same membrane but with lower signal (**A**). Quantitative analysis of c-FLIP Long (**B**), c-FLIP p43 high (**C**), c-FLIP p43 low (**D**), c-FLIP Short (**E**) and c-FLIP Raji (**F**) at different time points represented as fold change of protein levels compared to untreated control. Results are shown as average of five donors for LPS and three donors for TNF-α. Standard deviations are indicated. Asterisk denotes statistical significance as compared to the untreated control ($p < 0.05$, *One-way ANOVA with Bonferroni posttest*).

3.5 LPS-pretreatment increases birinapant-induced apoptosis in human macrophages

RIPK1 coordinates pyroptosis, apoptosis and necroptosis (referred to as PANoptosis) dependent and independent of its kinase activity [37, 93]. After having established that pretreatment of LPS before stimulation with birinapant reduced the cell viability and increased IL-1β release in macrophages, we wanted to investigate the mode of cell death induced. Macrophages with or without LPS-pretreatment overnight were stimulated with birinapant for 6 hours, lysed and immunoblotted. zVAD-fmk (zVAD), a pan-caspase inhibitor, was included as a positive control for necroptosis markers. zVAD inhibits apoptosis through irreversible binding of caspases, including caspase-3 and caspase-8, which results in necroptosis [70, 94]. A preliminary dose titration experiment showed that 4 μM of birinapant induced robust levels of cell death markers, and this was therefore chosen as treatment dose.

Immunoblotting showed that birinapant induced cleavage of the apoptotic protein caspase-8 at 18 kDa in macrophages treated with only birinapant (Figure 3.5). The levels of cleaved caspase-8 increased when the macrophages were pretreated with LPS and stimulated with birinapant.
The cleaved 17 kDa product of caspase-3 was observed in birinapant-treated macrophages (Figure 3.5). When the macrophages were pretreated with LPS prior to birinapant stimulation, the p17 levels of caspase-3 increased. Together, this indicates that birinapant induces apoptosis, and that pretreatment with LPS strengthens the apoptotic response in birinapant stimulated macrophages.

Kinase activation of RIPK1 and RIPK3 activates necroptosis [95]. Phosphorylation at Ser166 of RIPK1 and Ser227 of RIPK3 are markers of necroptosis, and we used these to test whether birinapant induced necroptosis with or without LPS-pretreatment. We included zVAD as a positive control for necroptosis. Birinapant induced a band at the expected molecular weight for phosphorylated RIPK1 at residue Ser166, but this was also somewhat present in the untreated samples. The band increased when macrophages pretreated with LPS and stimulated with birinapant were co-treated with zVAD (Figure 3.5). Also, the levels of phosphorylated RIPK3 at residue Ser227 increased when zVAD treatment was given to LPS-pretreated macrophages stimulated with birinapant, although weaker (Figure 3.5). Birinapant stimulation with or without LPS-pretreatment showed no phosphorylation of RIPK3. As a result, this indicates that LPS-pretreated macrophages stimulated with birinapant can induce necroptosis,

but not by itself. When caspases are blocked, these cells induce necroptosis-dependent cell death.

Cleavage of GSDMD at its N-terminal by caspase-1 or caspase-8 results in activation of GSDMD p30 and execution of pyroptosis. The inactive GSDMD p20 protein is also cleaved by these caspases. Caspase-3 cleavage of GSDMD leads to the formation of the inactive GSDMD p43 product and inhibition of pyroptosis [83, 96]. Both apoptosis and pyroptosis are dependent on caspases [97]. Immunoblotting showed that LPS-pretreatment increased the levels of GSDMD p43 in birinapant stimulated macrophages (Figure 3.5). This cleavage was caspase dependent as zVAD blocked cleavage. Neither the active GSDMD p30 protein nor GSDMD p20 were observed. Observation of cleaved caspase-3 and GSDMD p43 demonstrated that the protein levels followed the same trend. This suggest that caspase-3 is responsible for GSDMD cleavage.

LPS induced a higher-molecular variant of GSDMD, suggesting that LPS induce a modification of GSDMD (Figure 3.5, *). In addition, we observed high-molecular bands of GSDMD showing another pattern of modification than at the expected molecular size (Figure 3.5, **)

We also tested whether we could detect caspase-1 and IL-1β processing in the lysates. We did not follow up on these further as no bands were present in the samples.

Taken together, there were indications that apoptosis is the predominant mode for LPS-pretreated human macrophages stimulated with birinapant.

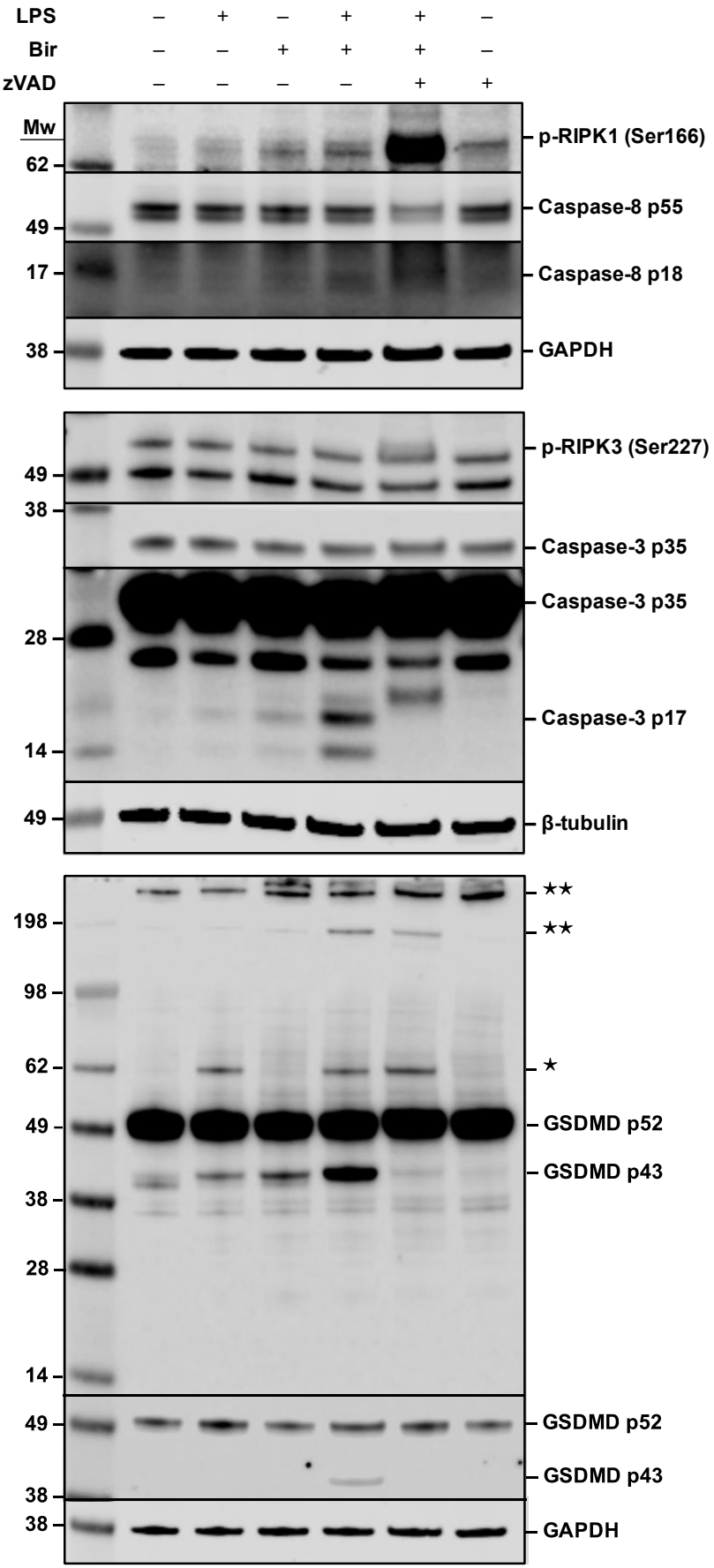


Figure 3.5 LPS-pretreatment increases birinapant-induced apoptosis in macrophages. Human macrophages were pretreated with 50 ng/mL LPS o/n (15-20 hours), before cells were washed and stimulated with either 4 µM birinapant, 20 µM zVAD or both for 6 hours. Cell lysates were analyzed for p-RIPK1 (Ser166), Caspase-8, p-RIPK3 (Ser227), Caspase-3 and GSDMD by immunoblotting. Single and double asterisk indicate unknown higher-molecular variants of GSDMD. One representative of five donors is shown. Immunoblots of all donors are found in Supplementary Figure S2. Qualitatively analysis was performed.

4 Discussion

The aim of this thesis was to investigate if the length of the pretreatment affected the activity of human macrophages by potentiating birinapant-induced cell death, and to test the mode of cell death induced. This was performed by pretreating human macrophages in a time series with either LPS or TNF-α before the cells were stimulated with birinapant. In addition to birinapant, LCL-161 was included to test if the effect was specific for birinapant or a general effect for SMs. Pretreatment of LPS and stimulation with birinapant reduced the viability of human macrophages, and short pretreatment was sufficient. LCL-161 did not reduce the viability of human macrophages after LPS-pretreatment, indicating that birinapant was more potent than LCL-161 to reduce cell viability in LPS-pretreated cells. Further studies revealed that pretreatment with TNF-α did not potentiate the cytotoxicity of birinapant. This suggests that the effect of LPS is specific in macrophages and potentiates birinapant cytotoxicity.

LPS-pretreated human macrophages stimulated with birinapant showed some IL-1β production as compared to LPS-treated. Further studies revealed the presence of caspase-8 cleavage product p18 in these macrophages suggesting caspase-8-dependent IL-1β production. We also observed caspase-3 and GDSMD p43 cleavage with this treatment indicating that these cells may be involved in apoptosis, and not pyroptosis. Protein levels of c-FLIP Long, Short, Raji and p43 high were found to be present with a significant amount in macrophages pretreated with LPS for 6 hours, proposing that LPS has a specific effect on increasing c-FLIP levels. Regarding mode of cell death, our findings suggest that LPS-pretreated macrophages stimulated with birinapant mainly induced apoptotic cell death.

4.1 LPS-pretreatment potentiates the cytotoxicity of birinapant-induced cell death in human macrophages

Preliminary studies performed by members of our group showed that the cytotoxicity of birinapant-induced cell death increased with 18 hours of LPS-pretreatment in macrophages (Klaharn 2019, *Unpublished*). Our results demonstrated that LPS potentiated the cytotoxicity of birinapant as early as after 5 minutes of pretreatment, but 6 hours of LPS-pretreatment was needed for a significant effect. Although not significant, there was a clear trend of reduction in cell viability at all time points when human macrophages were pretreated with LPS and stimulated with birinapant. This shows the importance of time series stimulation, as effect of

pretreatment varies over time. Donor variation in the control might be the reason why we did not get statistical significance for shorter pretreatment of LPS. However, this shows that pretreatment of LPS can be reduced from 18 to 6 hours to potentiate birinapant cytotoxicity.

Birinapant stimulated human macrophages were also pretreated with TNF-α in this study but showed not a potentiating effect on birinapant to induce cell death. This work therefore suggests that the effect is not due to a general activation of NF-κB/MAPK, but that the LPS effect is specific. It also proposes that this bacterial ligand performs as a switch-like activator to potentiate birinapant-induced cell death. Covert et al. displayed a non-oscillatory dynamic of active NF-κB in cells when stimulated with LPS, while TNF-α stimulated cells have been observed to show a damped oscillation of NF-κB activity [98]. This difference in NF-κB activation may also be an explanation for the different responses of LPS and TNF-α we observed. Further studies of NF-κB activation need to be conducted to determine this.

Birinapant induces RIPK1-dependent cell death via TLR4 and TNFR1 [44]. Our results demonstrated that only pretreatment with LPS, and not TNF-α, potentiated the effect of birinapant to induce cell death in human macrophages. This may suggest that LPS is a stronger inducer of apoptosis than TNF-α. A reason might be that TNF-α is a cytokine produced by the cell itself, while LPS is a substance originating from another organism. A critical task for the immune system is the ability to recognize between self and non-self. Since TNF-α is part of this system it might be that the immune reaction is damped compared to LPS in order to not induce a false alarm. TNF-α might have another main task than to induce cell death, such as paracrine and endocrine signaling or promoting inflammation. Xaus et al. demonstrated that stimulation with LPS induced autocrine secretion of TNF-α which resulted in early apoptotic events (3-6 hours). However, they also showed that LPS stimulation resulted in nitric oxide (NO) production which contributed to the late phase of apoptosis (12-24 hours) [99]. It may be that extracellular stimulation of TNF-α provides another reaction in the cell than autocrine signaling. And that NO has a prolonged effect and therefore plays a more central role in LPS-mediated apoptosis. Studies of NO and TNF-α production by performing ELISA could be conducted to further investigate their role in cell death.

Stimulation of LPS on human macrophages activates TLR4 signaling resulting in NF-κB/MAPK activation [43]. Depending on the length of stimulation, the activation results in

transcription of different intracellular proteins, such as IκBα and TNF-α mRNA expression [100]. This will also affect the macrophages to be in different states depending on how long they were stimulated with LPS. Our results showed that LPS-pretreatment for 6 hours significantly enhanced the cytotoxicity of birinapant. It would be interesting to further investigate which proteins are up- or downregulated by NF-κB/MAPK signaling after 6 hours of stimulation. This may give us indications of how LPS potentiates birinapant in human macrophages to induce cell death.

In this thesis two different SMs were tested to increase cell death in LPS-pretreated human macrophages. Birinapant showed more potency in reducing cell viability than LCL-161, and this has also been seen on previous works in our group [101]. This may be due to stronger binding affinity of birinapant than LCL-161 to the cIAP proteins. The reason may be that birinapant is a bivalent SM binding to two AVPI-like binding motifs of the cIAP protein, while LCL-161 is monovalent only binding to one such motif. To test if the binding affinity of the SM is a consequence of how great LPS-pretreatment potentiates SM-induced cell death, further studies could be performed. The use of other monovalent and bivalent IAP-antagonists, such as GDC-0917 and HGS1029 respectively, could be one way to test the effect of binding affinity of SM-induced cell death in LPS-pretreated macrophages.

Working with primary cells can be a challenge due to donor variation. Macrophages display heterogeneity in their response and occur when macrophages differentiate from their progenitors. Genetic variation may partly be a reason for this variety and the genetic background of the donor can therefore be a critical variable [102]. The health status of the donor can also be a considerable variable as disease increase the amount of white blood cells in the donor´s blood and make the cells more sensitive to stimuli. Therefore, a large sample size is needed when working with macrophages and other primary cells to obtain reproducible findings which can be considered biologically robust. Birinapant treatment on macrophages showed different donor-to-donor variation depending on the pretreatment. The donor variation observed with LPS-pretreatment was low, while there was a great variation with TNF-α pretreatment. This suggests that LPS has a specific effect on the macrophages independent of the donors.

4.2 Increased c-FLIP levels may not be sufficient to inhibit SM-induced apoptosis

Preliminary data obtained from members of our group displayed increased levels of c-FLIP in cells more prone to apoptosis when LPS-pretreated for 18 hours (Klaharn 2019, *Unpublished*). This was surprising as c-FLIP is an anti-apoptotic protein [55]. We demonstrated that the protein levels of c-FLIP significantly increased for c-FLIP Long (L), Short (S), Raji (R) and p43-high in human macrophages treated with LPS for 6 hours. Either c-FLIP can act proapoptotic in these cells. Alternatively, macrophages upregulate c-FLIP as a gatekeeper of apoptosis during infection, but this gate is omitted by birinapant. Immunoblot analysis showed more prominent levels of c-FLIP(L) compared to the other isoforms, indicating that it may overshadow the ability of c-FLIP(S) to induce necroptotic cell death. It would be interesting to further study what happens to c-FLIP when cell death occurs and what mode of cell death was induced.

To investigate the role of c-FLIP in apoptosis the protein can either be experimentally removed or overexpressed. Either siRNA or CRISPR-Cas9 could be used to remove c-FLIP. By this we could investigate if macrophages without c-FLIP are more or less sensitive to LPS + birinapant, and whether this is apoptosis. However, primary cells are hard to manipulate with these methods and removing c-FLIP would be challenging. Alternatively, overexpressing c-FLIP by the use of mRNA transfection could determine the role of c-FLIP in macrophages when the cells are not LPS-pretreated. With this method we could investigate if macrophages with overexpressed c-FLIP are more prone to induce apoptosis.

Studies have shown that moderate protein levels of c-FLIP(L) has a pro-apoptotic effect in the cell, but works in an anti-apoptotic manner when present at high levels [57]. Our data demonstrated significant elevated levels of c-FLIP(L) in LPS-pretreated macrophages after 6 hours. Also 6 hours of pretreatment by LPS showed a significant reduction in cell viability when we stimulated the human macrophages with birinapant. This might indicate that apoptotic prone human macrophages have increased c-FLIP(L) levels, therefore suggesting pro-apoptotic features even at high levels. However, not many studies of c-FLIP are performed in primary cells and additional roles other than being involved in cell death are unknown. Additional immunoblotting where LPS-pretreated macrophages also are stimulated with birinapant could have been tested to further investigate the role of c-FLIP(L) if time was not restricted.

We performed immunoblotting of known apoptotic markers. The presence of full-length caspase-8 and its cleaved active product (p18) were observed in LPS-pretreated macrophages stimulated with birinapant. This indicates that c-FLIP(L) was not able to inhibit caspase-8 activation. Our finding is supported by literature demonstrating that endogenous c-FLIP(L) is unable to block IAP antagonist-induced caspase-8 activation [103]. This may be why we observed reduction in cell viability in LPS-pretreated macrophages stimulated with birinapant. LPS seems to increase the levels of c-FLIP, while the SM blocks the ability of c-FLIP to inhibit the caspase-8 activity which makes the macrophages proceed to apoptosis. Wang et al. demonstrated that stimulation with SM did not change the levels of c-FLIP [103]. Therefore, we know that LPS stimulation and not SM-treatment increased the c-FLIP levels in our experiment. As a result, it may seem that caspase-8 has the ability to execute apoptosis without restraint when the cell death is induced by SMs.

4.3 Birinapant induces apoptosis in LPS-pretreated human macrophages

Immunoblotting of caspase-8 showed prominent protein levels of the full-length p55 isoform in birinapant stimulated macrophages with and without LPS-pretreatment. We were able to detect the cleaved active caspase-8 p18 when macrophages were pretreated with LPS and stimulated with birinapant, although in modest levels. Detecting cleaved caspase-8 using the same antibody stock has also been a challenge to other members of our group for other experiments. However, we also observed full-length caspase-3 and cleaved active caspase-3 p17 when the birinapant stimulated macrophages were LPS-pretreated. Caspase-3 cleavage can be induced by caspase-8. Due to the presence of cleaved caspase-3 we assume that caspase-8 was responsible for its activation, indicating presence of active caspase-8 in the cell. Observation of the cleaved caspases in our macrophages stimulated with birinapant after LPS-pretreatment indicated that the cells proceed to apoptosis. A quantitative proteomic mass spectrometry (MS) study has earlier been performed by members of our group comparing protein levels in macrophages treated with LPS+IFN-γ (Moen 2018, *Unpublished*). They observed a minor increase in caspase-8 levels compared to other apoptotic proteins.

In our immunoblot analysis we used zVAD, a pan-caspase inhibitor, as a positive control for necroptosis. Phosphorylation of RIPK1 was not present in birinapant stimulated macrophages with and without LPS-pretreatment. Addition of zVAD increased the levels of p-RIPK1 in macrophages stimulated with birinapant after pretreatment with LPS, indicating necroptotic cell

death. This demonstrates the PANoptosis crosstalk between the cell death pathways. Apoptosis seems to be the primary mode of cell death in these macrophages, but they can also proceed to necroptosis. However, necroptosis was not induced spontaneously by birinapant in LPS-pretreated macrophages but was obtained when apoptosis was blocked.

Earlier finding in our group showed that birinapant induced IL-1β production in macrophages after LPS-pretreatment for 18 hours (Klaharn 2019, *Unpublished*). In our work, 5 minutes of LPS-pretreatment induced IL-1β production in macrophages stimulated with birinapant. However, statistical analysis showed no significance when comparing the combinational treatment to untreated macrophages, nor birinapant alone. Comparisons between the treatments within each time point showed no significance either. We observed variance in the data for negative controls, where two donors displayed IL-1β secretion in untreated cells. There was also variance between LPS-pretreated macrophages stimulated with birinapant. Due to time constraints, we did not continue these experiments with more donors.

Madej et al. demonstrated that macrophages treated with LPS induce IL-1β production [104]. Our data suggests the opposite effect because some of our untreated macrophages displayed IL-1β production but when the cells were stimulated with LPS the production declined to about zero. This observation is consistent with the work of Netea et al. displaying no production of IL-1β in LPS-treated macrophages [105]. However, their findings also revealed that macrophages stimulated with LPS induced significantly amounts of precursor IL-1β mRNA, an effect also referred to as priming. This may be the reason why we observed IL-1β production when cell death was induced by birinapant in LPS-pretreated macrophages. LPS does not induce cell death by itself but prepares the macrophages to release IL-1β upon stimulation with birinapant. The cell changes it signaling from survival to death activating caspase-1 or caspase-8 which cleaves pro-IL-1β to mature IL-1β. Sagulenko et al. demonstrated that caspase-8 cleaves pro-IL-1β but the production of active IL-1β is very low as compared to caspase-1 [106]. This is consistent with our observation of present caspase-8 and findings of low IL-1β production. As a result, our study suggests that an additional stimulus, such as birinapant, is needed to produce and secrete IL-1β in LPS-pretreated human macrophages.

Cleaved GSDMD p30 translocate to the plasma membrane forming pores, releasing IL-1β and induces pyroptosis [79]. Our results demonstrated increased levels of cleaved GSDMD p43 in

LPS-pretreated macrophages stimulated with birinapant. This cleavage product induced by caspase-3 activity is known to inhibit pyroptosis. In compliance with modest IL-1β production performed by ELISA this indicates that macrophages with this treatment do not induce pyroptosis.

Caspase-1 has earlier been considered as the responsible caspase cleaving GSDMD to its active p30 pore-forming fragment which induces pyroptosis [83]. Studies performed by Orning et al. also showed caspase-8-directed cleavage of GSDMD p30 [107]. Our results displayed cleaved caspase-8 (p18) but no cleaved GSDMD p30. As we additionally find apoptosis activation but no signs of caspase-1 nor caspase-1 mediated GSDMD cleavage products, we suggest that caspase-8 induces apoptotic cell death in LPS-pretreated macrophages stimulated with birinapant. Macrophages with this cotreatment could be treated with Ac-YVAD-cmk, a caspase-1 inhibitor, to further investigate whether this would affect IL-1β release and GSDMD cleavage.

5 Conclusion and future perspectives

In this thesis we showed that pretreatment affect pharmaceutical targeting of cIAPs by SMs to increase birinapant-induced cell death in human macrophages *in vitro*. Our studies demonstrated that the cytotoxicity of birinapant was potentiated with LPS-pretreatment for 5 minutes in human macrophages, but 6 hours of pretreatment was needed for a significant effect. This was not observed for macrophages pretreated with the pro-inflammatory cytokine TNF-α, that also signals through RIPK1 and cIAP1/2. Also, c-FLIP protein levels increased in LPS-treated macrophages. The presence of cleaved caspase-3 and caspase-8 indicated that the primary mode of cell death induced by birinapant in LPS-pretreated macrophages was apoptosis. However, when this mode was blocked by zVAD necroptosis proceeded but not spontaneously. We excluded birinapant-induced pyroptosis in LPS-pretreated macrophages due to the presence of cleaved caspase-3 and GSDMD p43.

SMs have a potential to be used in inflammatory diseases, but studies in human macrophages are scarce. Jensen et al. outline the importance of testing SMs in human model systems as a crucial step for using SMs in the clinic to treat inflammatory diseases [27]. In our study we showed that short pretreatment with LPS potentiated the cytotoxicity of SMs to induce an effect on macrophage viability. Our studies demonstrated that birinapant reduced cell viability in LPS-pretreated human macrophages after 5 minutes, and the effect was significant after 6 hours of pretreatment. This is promising for treatment of diseases where overactive macrophages are central for disease development. Further studies testing other bivalent SMs, such as HGS1029, on LPS-pretreated human macrophages should be conducted to evaluate whether their cytotoxicity significantly increase with pretreatment, or if the effect was specific for birinapant. We also found increased levels of c-FLIP after 6 hours of LPS-pretreatment. It would be interesting to observe if overexpressed c-FLIP in human macrophages not treated with LPS are more prone to induce apoptosis. Our results displayed that caspase-8 was activated in our macrophages but no signs of caspase-1 were observed. Additional treatment with the caspase-1 inhibitor Ac-YVAD-cmk on LPS-pretreated human macrophages stimulated with birinapant should be conducted. It would be interesting to evaluate if caspase-1 inhibition would affect IL-1β release and GSDMD cleavage, and ELISA analysis and immunoblotting could be performed to test this. Additionally, the use of human macrophages in future studies of SMs are essential for the progress of SMs as therapeutic drugs to treat inflammatory diseases.

6 References

1. Italiani, P. & Boraschi, D. From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation. *Frontiers in Immunology* vol. 5 (2014).
2. Ginhoux, F. & Jung, S. Monocytes and macrophages: Developmental pathways and tissue homeostasis. *Nature Reviews Immunology* (2014) doi:10.1038/nri3671.
3. Shi, C. & Pamer, E. G. Monocyte recruitment during infection and inflammation. *Nature Reviews Immunology* (2011) doi:10.1038/nri3070.
4. Lin, W. Y. *et al.* Function of CSF1 and IL34 in Macrophage Homeostasis, Inflammation, and Cancer. *Front. Immunol.* **10**, 1–18 (2019).
5. Liu, Y. C., Zou, X. B., Chai, Y. F. & Yao, Y. M. Macrophage polarization in inflammatory diseases. *Int. J. Biol. Sci.* **10**, 520–529 (2014).
6. Murray, P. J. & Wynn, T. A. Protective and pathogenic functions of macrophage subsets. *Nature Reviews Immunology* (2011) doi:10.1038/nri3073.
7. Wynn, T. A., Chawla, A. & Pollard, J. W. Macrophage biology in development, homeostasis and disease. *Nature* (2013) doi:10.1038/nature12034.
8. Jones, C. V. & Ricardo, S. D. Macrophages and CSF-1: Implications for development and beyond. *Organogenesis* **9**, 249–260 (2013).
9. Murray, P. J. *et al.* Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity* **41**, 14–20 (2014).
10. Isidro, R. A. & Appleyard, C. B. Colonic macrophage polarization in homeostasis, inflammation, and cancer. *Am. J. Physiol. - Gastrointest. Liver Physiol.* **311**, G59–G73 (2016).
11. Chistiakov, D. A., Myasoedova, V. A., Revin, V. V., Orekhov, A. N. & Bobryshev, Y. V. The impact of interferon-regulatory factors to macrophage differentiation and polarization into M1 and M2. *Immunobiology* (2018) doi:10.1016/j.imbio.2017.10.005.
12. Wang, N., Liang, H. & Zen, K. Molecular mechanisms that influence the macrophage M1-M2 polarization balance. *Front. Immunol.* **5**, 1–9 (2014).
13. Guttman, O. & C. Lewis, E. M2-like macrophages and tumor-associated macrophages: overlapping and distinguishing properties en route to a safe therapeutic potential. *Integr. Cancer Sci. Ther.* **3**, 554–561 (2016).
14. Vallabhapurapu, S. & Karin, M. Regulation and function of NF-κB transcription factors in the immune system. *Annu. Rev. Immunol.* **27**, 693–733 (2009).
15. Liu, T., Zhang, L., Joo, D. & Sun, S. C. NF-κB signaling in inflammation. *Signal*

Transduct. Target. Ther. **2**, (2017).

16. Pahl, H. L. Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* **18**, 6853–6866 (1999).
17. Boyce, B. F., Xiu, Y., Li, J., Xing, L. & Yao, Z. NF-κB-mediated regulation of osteoclastogenesis. *Endocrinol. Metab.* **30**, 35–44 (2015).
18. Taniguchi, K. & Karin, M. NF-kB, inflammation, immunity and cancer: Coming of age. *Nat. Rev. Immunol.* **18**, 309–324 (2018).
19. Hoffmann, A. & Baltimore, D. Circuitry of nuclear factor κB signaling. *Immunol. Rev.* **210**, 171–186 (2006).
20. Israël, A. The IKK complex, a central regulator of NF-kappaB activation. *Cold Spring Harb. Perspect. Biol.* **2**, 1–14 (2010).
21. Kanayama, A. *et al.* TAB2 and TAB3 activate the NF-κB pathway through binding to polyubiquitin chains. *Mol. Cell* **15**, 535–548 (2004).
22. Sakurai, H. *et al.* TAK1-TAB1 fusion protein: A novel constitutively active mitogen-activated protein kinase kinase kinase that stimulates AP-1 and NF-κB signaling pathways. *Biochem. Biophys. Res. Commun.* **297**, 1277–1281 (2002).
23. Ajibade, A. A., Wang, H. Y. & Wang, R. F. Cell type-specific function of TAK1 in innate immune signaling. *Trends Immunol.* **34**, 307–316 (2013).
24. Sun, S. C. The non-canonical NF-κB pathway in immunity and inflammation. *Nat. Rev. Immunol.* **17**, 545–558 (2017).
25. Sun, S. C. Non-canonical NF-κB signaling pathway. *Cell Res.* **21**, 71–85 (2011).
26. Kim, E. K. & Choi, E. J. Pathological roles of MAPK signaling pathways in human diseases. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1802**, 396–405 (2010).
27. Jensen, S., Seidelin, J. B., LaCasse, E. C. & Nielsen, O. H. SMAC mimetics and RIPK inhibitors as therapeutics for chronic inflammatory diseases. *Sci. Signal.* **13**, 1–18 (2020).
28. HuangFu, W. C., Omori, E., Akira, S., Matsumoto, K. & Ninomiya-Tsuji, J. Osmotic stress activates the TAK1-JNK pathway while blocking TAK1-mediated NF-κB activation: TAO2 regulates TAK1 pathways. *J. Biol. Chem.* **281**, 28802–28810 (2006).
29. Mihaly, S. R., Ninomiya-Tsuji, J. & Morioka, S. TAK1 control of cell death. *Cell Death Differ.* **21**, 1667–1676 (2014).
30. Cuchet-Lourenço, D. *et al.* Biallelic RIPK1 mutations in humans cause severe immunodeficiency, arthritis, and intestinal inflammation. *Science (80-.).* **361**, 810–813 (2018).

31. Ofengeim, D. & Yuan, J. Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. *Nat. Rev. Mol. Cell Biol.* **14**, 727–736 (2013).
32. Peltzer, N., Darding, M. & Walczak, H. Holding RIPK1 on the Ubiquitin Leash in TNFR1 Signaling. *Trends Cell Biol.* **26**, 445–461 (2016).
33. Degterev, A., Ofengeim, D. & Yuan, J. Targeting RIPK1 for the treatment of human diseases. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 9714–9722 (2019).
34. Schrader, E. K., Harstad, K. G. & Matouschek, A. Targeting proteins for degradation. *Nat. Chem. Biol.* **5**, 815–822 (2009).
35. Chen, R. H., Chen, Y. H. & Huang, T. Y. Ubiquitin-mediated regulation of autophagy. *J. Biomed. Sci.* **26**, 1–12 (2019).
36. Benetatos, C. A. *et al.* Birinapant (TL32711), a bivalent SMAC mimetic, targets TRAF2-associated cIAPs, abrogates TNF-induced NF-κβ activation, and is active in patient-derived xenograft models. *Mol. Cancer Ther.* **13**, 867–879 (2014).
37. Malireddi, R. K. S. *et al.* Innate immune priming in the absence of TAK1 drives RIPK1 kinase activity–independent pyroptosis, apoptosis, necroptosis, and inflammatory disease. *J. Exp. Med.* **217**, 1–13 (2020).
38. Hunter, A. M., LaCasse, E. C. & Korneluk, R. G. The inhibitors of apoptosis (IAPs) as cancer targets. *Apoptosis* (2007) doi:10.1007/s10495-007-0087-3.
39. Morrish, E., Brumatti, G. & Silke, J. Future Therapeutic Directions for Smac-Mimetics. *Cells* **9**, 406 (2020).
40. Guicciardi, M. E. *et al.* Cellular inhibitor of apoptosis 1 (cIAP-1) degradation by caspase 8 during TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. *Exp. Cell Res.* **317**, 107–116 (2011).
41. Condon, S. M. *et al.* Birinapant, a smac-mimetic with improved tolerability for the treatment of solid tumors and hematological malignancies. *J. Med. Chem.* **57**, 3666–3677 (2014).
42. Ciesielska, A., Matyjek, M. & Kwiatkowska, K. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cell. Mol. Life Sci.* **78**, 1233–1261 (2021).
43. Into, T., Inomata, M., Takayama, E. & Takigawa, T. Autophagy in regulation of Toll-like receptor signaling. *Cell. Signal.* **24**, 1150–1162 (2012).
44. Liu, L. & Lalaoui, N. 25 years of research put RIPK1 in the clinic. *Semin. Cell Dev. Biol.* (2020) doi:10.1016/j.semcdb.2020.08.007.
45. Hu, H. & Sun, S. C. Ubiquitin signaling in immune responses. *Cell Res.* **26**, 457–483

(2016).

46. Wertz, I. E. *et al.* ligase domains of A20 downregulate NF- k B signalling. *Nature* **430**, 1–6 (2004).
47. Lalaoui, N. *et al.* Targeting triple-negative breast cancers with the Smac-mimetic birinapant. *Cell Death Differ.* **27**, 2768–2780 (2020).
48. Guo, X. *et al.* TAK1 regulates caspase 8 activation and necroptotic signaling via multiple cell death checkpoints. *Cell Death Dis.* **7**, 1–11 (2016).
49. Galluzzi, L. *et al.* Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ.* **25**, 486–541 (2018).
50. Elmore, S. Apoptosis: A Review of Programmed Cell Death. *Toxicol. Pathol.* **35**, 495–516 (2007).
51. Chen, K. W. *et al.* Extrinsic and intrinsic apoptosis activate pannexin-1 to drive NLRP 3 inflammasome assembly . *EMBO J.* **38**, 1–12 (2019).
52. Lin, H. H. *et al.* Activation of apoptosis by Salmonella pathogenicity island-1 effectors through both intrinsic and extrinsic pathways in Salmonella-infected macrophages. *J. Microbiol. Immunol. Infect.* (2020) doi:10.1016/j.jmii.2020.02.008.
53. Slack, J. M. W. Molecular Biology of the Cell. in *Principles of Tissue Engineering: Fourth Edition* (2013). doi:10.1016/B978-0-12-398358-9.00007-0.
54. Man, S. M., Karki, R. & Kanneganti, T. D. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. *Immunol. Rev.* **277**, 61–75 (2017).
55. Telieps, T. *et al.* Cellular-FLIP, Raji isoform (c-FLIPR) modulates cell death induction upon T-cell activation and infection. *Eur. J. Immunol.* **43**, 1499–1510 (2013).
56. Busca, A., Saxena, M., Kryworuchko, M. & Kumar, A. Anti-Apoptotic Genes in the Survival of Monocytic Cells During Infection. *Curr. Genomics* **10**, 306–317 (2009).
57. Hillert, L. K. *et al.* Dissecting DISC regulation via pharmacological targeting of caspase-8/c-FLIPL heterodimer. *Cell Death Differ.* **27**, 2117–2130 (2020).
58. Scaffidi, C., Schmitz, I., Krammer, P. H. & Peter, M. E. The role of c-FLIP in modulation of CD95-induced apoptosis. *J. Biol. Chem.* **274**, 1541–1548 (1999).
59. Kataoka, T. & Tschopp, J. N-Terminal Fragment of c-FLIP(L) Processed by Caspase 8 Specifically Interacts with TRAF2 and Induces Activation of the NF-κB Signaling Pathway. *Mol. Cell. Biol.* **24**, 2627–2636 (2004).
60. Humphreys, L., Espona-Fiedler, M. & Longley, D. B. FLIP as a therapeutic target in cancer. *FEBS J.* **285**, 4104–4123 (2018).

61. Smyth, P., Sessler, T., Scott, C. J. & Longley, D. B. FLIP(L): the pseudo-caspase. *FEBS J.* **287**, 4246–4260 (2020).
62. Telieps, T. *et al.* Cellular-FLIP, Raji isoform (c-FLIPR) modulates cell death induction upon T-cell activation and infection. *Eur. J. Immunol.* **43**, 1499–1510 (2013).
63. Yang, Z. H. *et al.* A Non-canonical PDK1-RSK Signal Diminishes Pro-caspase-8-Mediated Necroptosis Blockade. *Mol. Cell* **80**, 296-310.e6 (2020).
64. Tang, Y. *et al.* Linear ubiquitination of cFLIP induced by LUBAC contributes to TNF-induced apoptosis. *J. Biol. Chem.* **293**, 20062–20072 (2018).
65. Weir, A., Hughes, S., Rashidi, M., Hildebrand, J. M. & Vince, J. E. Necroptotic movers and shakers: cell types, inflammatory drivers and diseases. *Curr. Opin. Immunol.* **68**, 83–97 (2021).
66. Chen, W. *et al.* Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling. *J. Biol. Chem.* **288**, 16247–16261 (2013).
67. Varga, Z. *et al.* Differences in the sensitivity of classically and alternatively activated macrophages to TAK1 inhibitor-induced necroptosis. *Cancer Immunol. Immunother.* **69**, 2193–2207 (2020).
68. Roh, J. S. & Sohn, D. H. Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune Netw.* **18**, 1–14 (2018).
69. Chen, J., Kos, R., Garssen, J. & Redegeld, F. Molecular Insights into the Mechanism of Necroptosis: The Necrosome As a Potential Therapeutic Target. *Cells* **8**, 1–20 (2019).
70. Pasparakis, M. & Vandenabeele, P. Necroptosis and its role in inflammation. *Nature* **517**, 311–320 (2015).
71. Hildebrand, J. M. & Murphy, J. M. The Highway to Hell: A RIP Kinase-Directed Shortcut to Inflammatory Cytokine Production. *Immunity* **45**, 1–3 (2016).
72. Cai, Z. *et al.* Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. *Nat. Cell Biol.* **16**, 55–65 (2014).
73. Lawlor, K. E. *et al.* RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL. *Nat. Commun.* **6**, (2015).
74. Shi, J., Gao, W. & Shao, F. Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death. *Trends Biochem. Sci.* **42**, 245–254 (2017).
75. Broz, P. *et al.* Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *J. Exp. Med.* **207**, 1745–1755 (2010).
76. Man, S. M. & Kanneganti, T. D. Regulation of inflammasome activation. *Immunol.*

Rev. **265**, 6–21 (2015).

77. Sharma, D. & Kanneganti, T. D. The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. *J. Cell Biol.* **213**, 617–629 (2016).
78. Yang, Y., Wang, H., Kouadir, M., Song, H. & Shi, F. Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. *Cell Death Dis.* **10**, (2019).
79. Liu, X. *et al.* Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* **535**, 153–158 (2016).
80. Sarhan, J. *et al.* Caspase-8 induces cleavage of gasdermin D to elicit pyroptosis during Yersinia infection. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E10888–E10897 (2018).
81. Man, S. M. *et al.* Salmonella Infection Induces Recruitment of Caspase-8 to the Inflammasome To Modulate IL-1β Production . *J. Immunol.* **191**, 5239–5246 (2013).
82. Gurung, P. *et al.* FADD and Caspase-8 Mediate Priming and Activation of the Canonical and Noncanonical Nlrp3 Inflammasomes. *J. Immunol.* **192**, 1835–1846 (2014).
83. Taabazuing, C. Y., Okondo, M. C. & Bachovchin, D. A. Pyroptosis and Apoptosis Pathways Engage in Bidirectional Crosstalk in Monocytes and Macrophages. *Cell Chem. Biol.* **24**, 507-514.e4 (2017).
84. Tao, P. *et al.* A dominant autoinflammatory disease caused by non-cleavable variants of RIPK1. *Nature* **577**, 109–114 (2020).
85. Derakhshan, A., Chen, Z. & Van Waes, C. Therapeutic small molecules target inhibitor of apoptosis proteins in cancers with deregulation of extrinsic and intrinsic cell death pathways. *Clin. Cancer Res.* **23**, 1379–1387 (2017).
86. Li, Y. *et al.* Human RIPK1 deficiency causes combined immunodeficiency and inflammatory bowel diseases. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 970–975 (2019).
87. Varfolomeev, E. *et al.* c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor α (TNFα)-induced NF-κB activation. *J. Biol. Chem.* **283**, 24295–24299 (2008).
88. Varfolomeev, E. *et al.* IAP Antagonists Induce Autoubiquitination of c-IAPs, NF-κB Activation, and TNFα-Dependent Apoptosis. *Cell* **131**, 669–681 (2007).
89. Fulda, S. Promises and challenges of Smac mimetics as cancer therapeutics. *Clin. Cancer Res.* **21**, 5030–5036 (2015).
90. Ali, H. *et al.* Selective killing of human M1 macrophages by Smac mimetics alone and M2 macrophages by Smac mimetics and caspase inhibition. *J. Leukoc. Biol.* (2021) doi:10.1002/JLB.4A0220-114RR.

91. Udalova, I. A., Mantovani, A. & Feldmann, M. Macrophage heterogeneity in the context of rheumatoid arthritis. *Nat. Rev. Rheumatol.* **12**, 472–485 (2016).
92. Decker, T. & Lohmann-Matthes, M. L. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Methods* **115**, 61–69 (1988).
93. Karki, R. *et al.* Synergism of TNF-α and IFN-γ Triggers Inflammatory Cell Death, Tissue Damage, and Mortality in SARS-CoV-2 Infection and Cytokine Shock Syndromes. *Cell* **184**, 149-168.e17 (2021).
94. Philip, N. H. *et al.* Caspase-8 mediates caspase-1 processing and innate immune defense in response to bacterial blockade of NF- B and MAPK signaling. *Proc. Natl. Acad. Sci.* **111**, 7385–7390 (2014).
95. Cho, Y. S. *et al.* Phosphorylation-Driven Assembly of the RIP1-RIP3 Complex Regulates Programmed Necrosis and Virus-Induced Inflammation. *Cell* **137**, 1112–1123 (2009).
96. Demarco, B. *et al.* Caspase-8-dependent gasdermin D cleavage promotes antimicrobial defense but confers susceptibility to TNF-induced lethality. *Sci. Adv.* **6**, (2020).
97. Miao, E. A., Rajan, J. V. & Aderem, A. Caspase-1-induced pyroptotic cell death. *Immunol. Rev.* **243**, 206–214 (2011).
98. Covert, M. W., Leung, T. H., Gaston, J. E. & Baltimore, D. Achieving stability of lipopolysaccharide-induced NF-κB activation. *Science (80-.).* **309**, 1854–1857 (2005).
99. Xaus, J. *et al.* LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-α. *Blood* **95**, 3823–3831 (2000).
100. Hobbs, S., Reynoso, M., Geddis, A. V., Mitrophanov, A. Y. & Matheny, R. W. LPS-stimulated NF-κB p65 dynamic response marks the initiation of TNF expression and transition to IL-10 expression in RAW 264.7 macrophages. *Physiol. Rep.* **6**, 1–16 (2018).
101. Moen, I. N. *et al.* Smac-mimetics reduce numbers and viability of human osteoclasts. *Cell Death Discov.* **7**, 1–11 (2021).
102. Sharif, O., Bolshakov, V. N., Raines, S., Newham, P. & Perkins, N. D. Transcriptional profiling of the LPS induced NF-κB response in macrophages. *BMC Immunol.* **8**, 1–17 (2007).
103. Wang, L., Du, F. & Wang, X. TNF-α Induces Two Distinct Caspase-8 Activation Pathways. *Cell* **133**, 693–703 (2008).
104. Madej, M. P., Töpfer, E., Boraschi, D. & Italiani, P. Different regulation of interleukin-

1 production and activity in monocytes and macrophages: Innate memory as an endogenous mechanism of IL-1 inhibition. *Front. Pharmacol.* **8**, 1–12 (2017).

105. Netea, M. G. *et al.* Differential requirement for the activation of the inflammasome for processing and release of IL-1β in monocytes and macrophages. *Blood* **113**, 2324–2335 (2009).
106. Sagulenko, V. *et al.* AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell Death Differ.* **20**, 1149–1160 (2013).
107. Orning, P. *et al.* Pathogen blockade of TAK1 triggers caspase-8–dependent cleavage of gasdermin D and cell death. *Science (80-.).* **362**, 1064–1069 (2018).

7 Supplementary

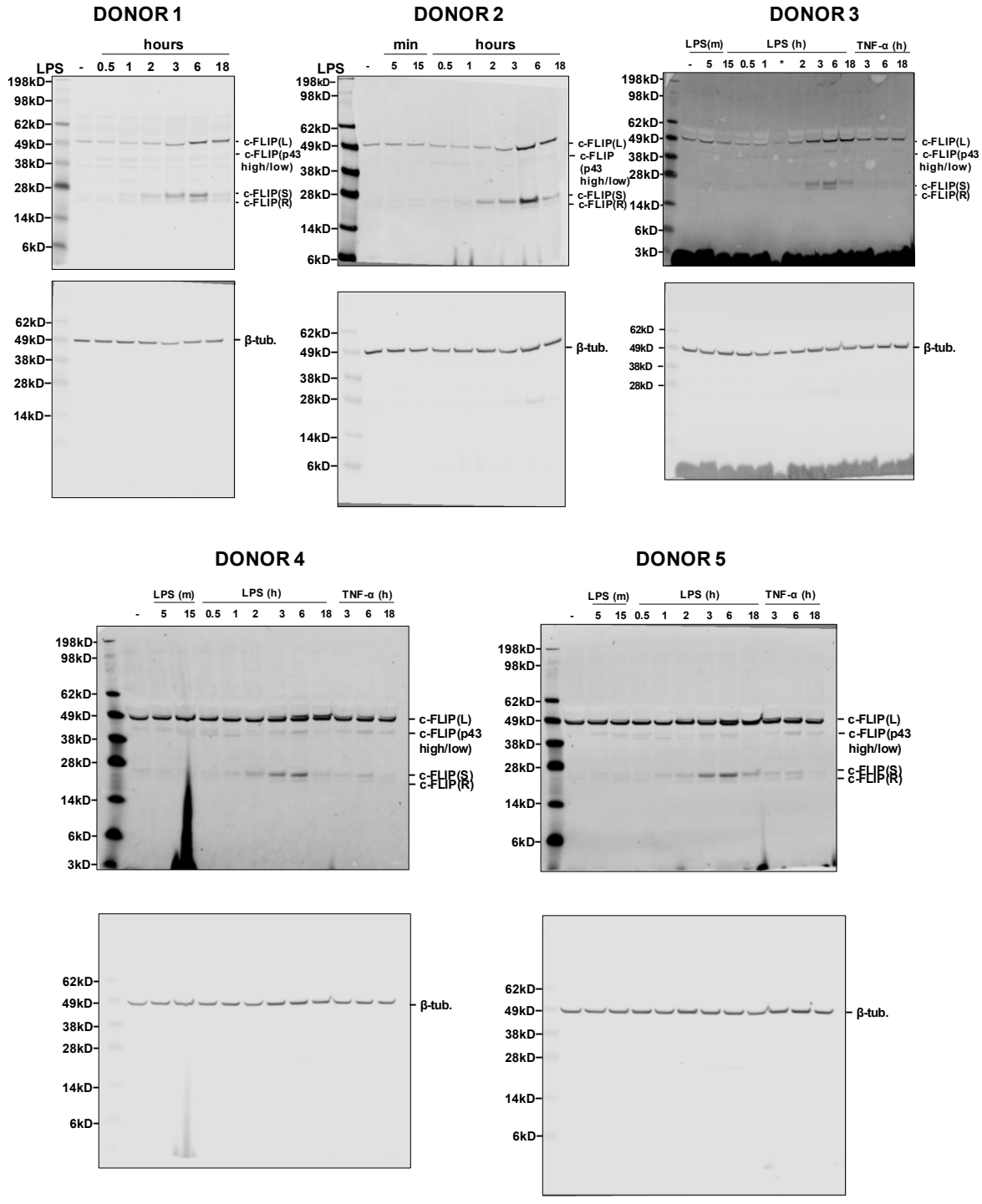
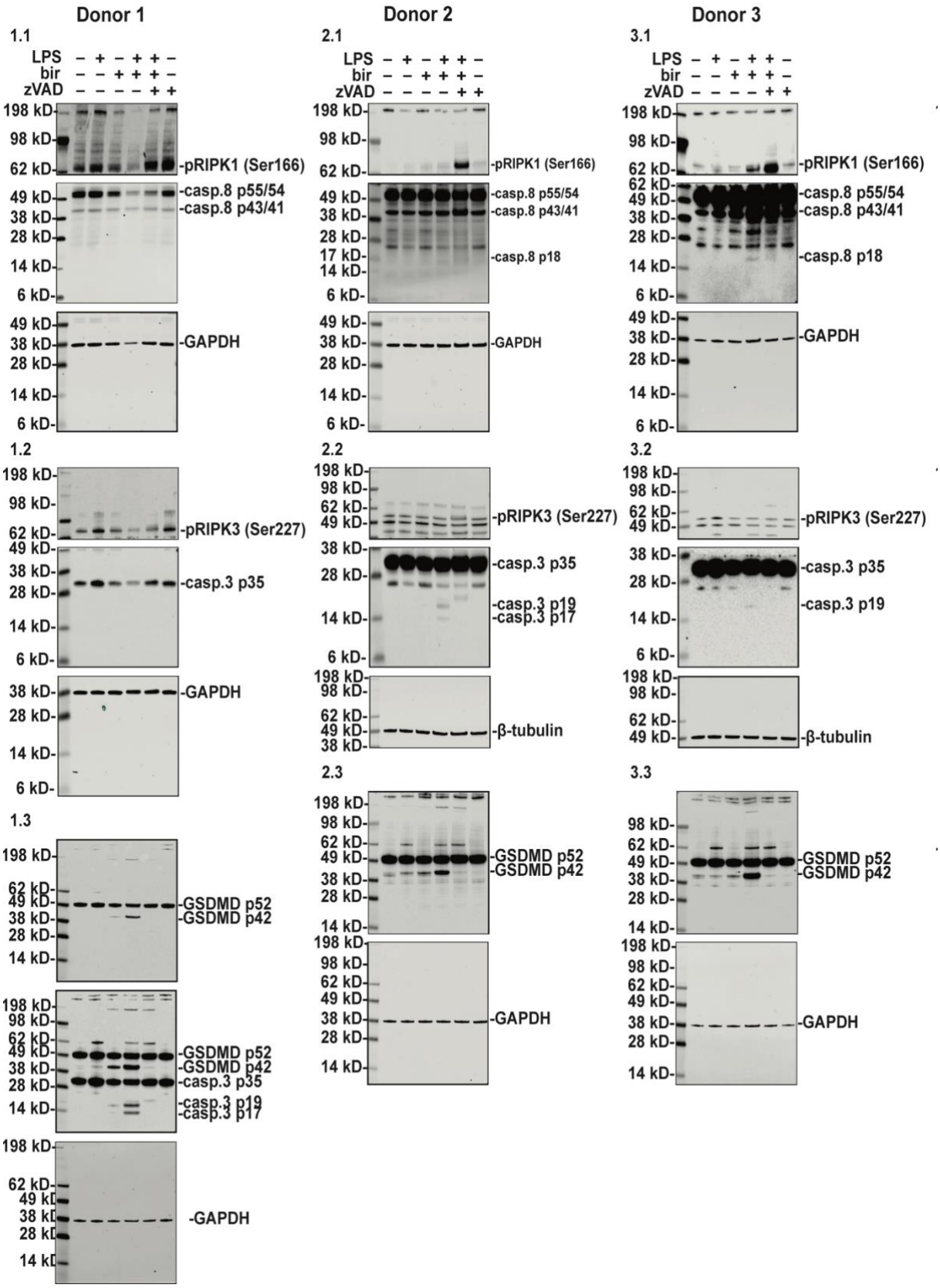


Figure S1 Donors and full immunoblots of c-FLIP for LPS and TNF-α-treatment of human macrophages. Human macrophages were treated with 50 ng/mL LPS or 1 ng/mL TNF-α at indicated time points. Cell lysates were analyzed for c-FLIP isoform proteins and cleaved products by immunoblotting. β-tubulin is used as loading control. Donor 5 is the same as in Figure 3.4.


Donor 1
Donor 2
Donor 3
1.1
2.1
3.1
LPS
bir
zVAD
198 kD
98 kD
62 kD
49 kD
38 kD
28 kD
17 kD
14 kD
6 kD
-pRIPK1 (Ser166)
-casp.8 p55/54
-casp.8 p43/41
-casp.8 p18
-GAPDH
1.2
2.2
3.2
-pRIPK3 (Ser227)
-casp.3 p35
-casp.3 p19
-casp.3 p17
-β-tubulin
1.3
2.3
3.3
-GSDMD p52
-GSDMD p42

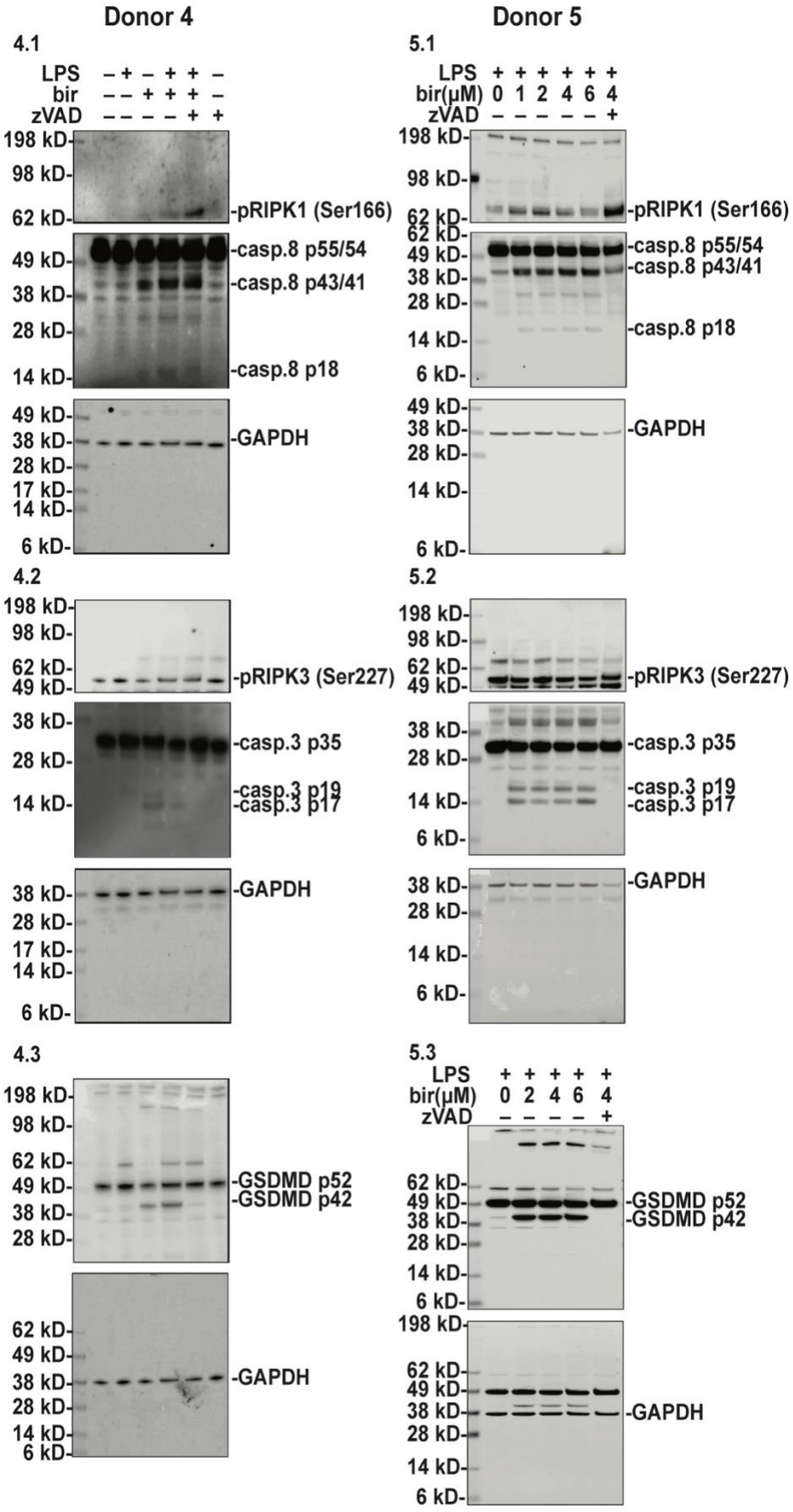
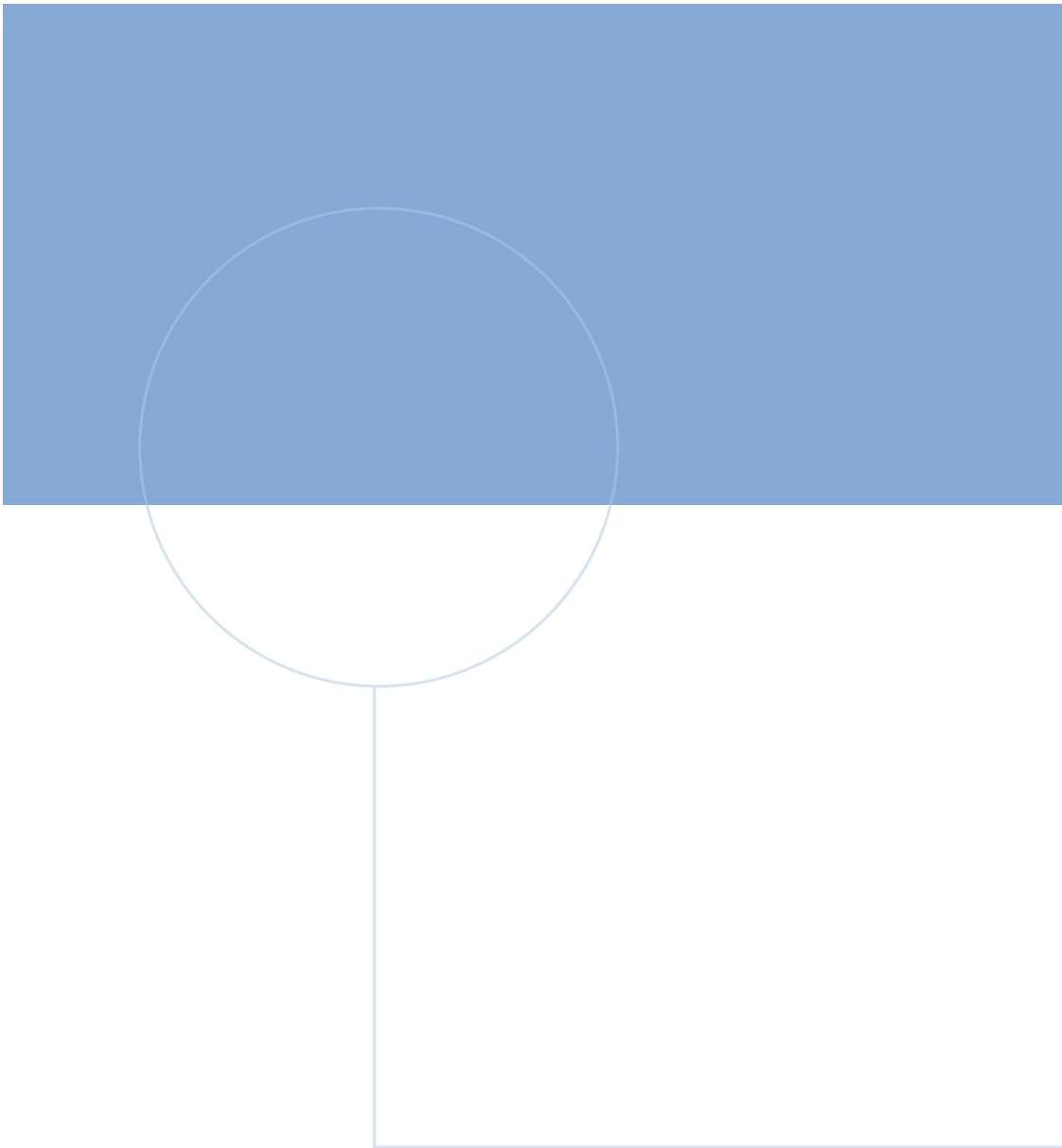

Donor 4
4.1
LPS – + – + + –
bir – – + + + –
zVAD – – – – + +
198 kD-
98 kD-
62 kD-
-pRIPK1 (Ser166)
49 kD-
38 kD-
28 kD-
14 kD-
-casp.8 p55/54
-casp.8 p43/41
-casp.8 p18
49 kD-
38 kD-
28 kD-
17 kD-
14 kD-
6 kD-
-GAPDH
4.2
198 kD-
98 kD-
62 kD-
49 kD-
-pRIPK3 (Ser227)
38 kD-
28 kD-
14 kD-
-casp.3 p35
-casp.3 p19
-casp.3 p17
38 kD-
28 kD-
17 kD-
14 kD-
6 kD-
-GAPDH
4.3
198 kD-
98 kD-
62 kD-
49 kD-
38 kD-
28 kD-
-GSDMD p52
-GSDMD p42
62 kD-
49 kD-
38 kD-
28 kD-
14 kD-
6 kD-
-GAPDH
Donor 5
5.1
LPS + + + + + +
bir(μM) 0 1 2 4 6 4
zVAD – – – – – +
198 kD-
98 kD-
62 kD-
-pRIPK1 (Ser166)
62 kD-
49 kD-
38 kD-
28 kD-
14 kD-
6 kD-
-casp.8 p55/54
-casp.8 p43/41
-casp.8 p18
49 kD-
38 kD-
28 kD-
14 kD-
6 kD-
-GAPDH
5.2
198 kD-
98 kD-
62 kD-
49 kD-
-pRIPK3 (Ser227)
38 kD-
28 kD-
14 kD-
6 kD-
-casp.3 p35
-casp.3 p19
-casp.3 p17
38 kD-
28 kD-
14 kD-
6 kD-
-GAPDH
5.3
LPS + + + + +
bir(μM) 0 2 4 6 4
zVAD – – – – +
62 kD-
49 kD-
38 kD-
28 kD-
14 kD-
6 kD-
-GSDMD p52
-GSDMD p42
198 kD-
62 kD-
49 kD-
38 kD-
28 kD-
14 kD-
6 kD-
-GAPDH

Figure S2 Donors and full immunoblots for human macrophages pretreated with LPS and stimulated with birinapant and zVAD. Human macrophages were pretreated with 50 ng/mL LPS o/n (15-20 hours), before cells were washed and stimulated with either birinapant, 20 µM zVAD or both for 6 hours. Donor 1-4 were treated with 4 µM birinapant, while donor 5 was treated with indicated concentrations of birinapant. Cell lysates were analyzed for p-RIPK1 (Ser166), Caspase-8, p-RIPK3 (Ser227), Caspase-3 and GSDMD by immunoblotting. Three membranes for each donor were used for immunoblotting. Immunoblots are designated with donor number and membrane number. GAPDH is used as loading control for donor 1-5, except membrane 2.2 and 3.2. Donor 2 is the same as in Figure 3.5. Qualitatively analysis was performed.



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