1-18-2018

The Role of RIPK1 Kinase Activity in Regulating Inflammation and Necroptotic Death

Matija Zelic
University of Massachusetts Medical School

Follow this and additional works at: https://escholarship.umassmed.edu/gsbs_diss
Part of the Animal Experimentation and Research Commons, and the Other Immunology and Infectious Disease Commons

This work is licensed under a Creative Commons Attribution 4.0 License.

Repository Citation

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
THE ROLE OF RIPK1 KINASE ACTIVITY IN REGULATING
INFLAMMATION AND NECROPTOTIC DEATH

A Dissertation Presented
By
MATIJA ZELIC

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

JANUARY 18, 2018
MOLECULAR, CELL AND CANCER BIOLOGY
THE ROLE OF RIPK1 KINASE ACTIVITY IN REGULATING INFLAMMATION AND NECROPTOTIC DEATH

A Dissertation Presented
By

MATIJA ZELIC

This work was undertaken in the Graduate School of Biomedical Sciences

Molecular, Cell and Cancer Biology

Under the mentorship of

____________________________
Michelle Kelliher, Ph.D., Thesis Advisor

____________________________
Katherine Fitzgerald, Ph.D., Member of Committee

____________________________
Egil Lien, Ph.D., Member of Committee

____________________________
Neal Silverman, Ph.D., Member of Committee

____________________________
Alexei Degterev, Ph.D., External Member of Committee

____________________________
Eric Baehrecke, Ph.D., Chair of Committee

____________________________
Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

January 18, 2018
Acknowledgments

I would like to thank my mentor, Michelle Kelliher, for letting me join her lab to pursue interesting research and for her guidance and support throughout my graduate school studies. Additionally, I would like to thank my committee members, Eric Baehrecke, Kate Fitzgerald, Egil Lien, Neal Silverman, Alexei Degterev, and Stephen Jones, for their advice, support and constructive criticisms. I am grateful to all members of the Kelliher Lab, past and present, for their friendship and assistance, and for helping to create a fun and supportive lab environment. I would especially like to thank Nicole Hermance, Justine Roderick, Jo O’Donnell, and Jesse Lehman for all of their assistance, especially with experiments and data analysis presented in my thesis. I would also like to thank Chinmay Trivedi, and his graduate student Harish Palleti Janardhan, for their assistance and support with the liver immunohistochemistry in chapter 4, and Egil Lien and Pontus Orning for providing and helping prepare the LPS used for endotoxemia studies in chapter 3.

I would not be where I am without the constant support and encouragement of my family – my parents, my grand-parents, and my brother Roko. Thank you for instilling in me important values like hard work, and for the love you have always provided. My girlfriend, fiancé, and now wife, Jen, who has been on this whole graduate school journey with me from the beginning, I can’t thank you enough for always being there for me. It’s comforting to know I’ll always have someone to drink a beer with.
Abstract

Necroptosis, a type of regulated necrotic cell death, involves cell membrane permeabilization and has been implicated in various acute and chronic pro-inflammatory diseases, including ischemia-reperfusion injury and neurodegenerative diseases. By using in vitro reconstitution studies and a chemical inhibitor, the kinase activity of the serine/threonine kinase RIPK1 had been shown to regulate necroptotic signaling downstream of TNF and Toll-like receptors (TLRs). To investigate the contribution of RIPK1 kinase activity to inflammation and necroptosis in vivo, we generated kinase inactive RIPK1 knock-in mice. Utilizing fibroblasts and macrophages from these mice, we demonstrate that RIPK1 kinase activity is required for necroptotic complex formation and death induction downstream of TNFR1 and TLRs 3 and 4. We show that RIPK1 kinase inactive mice are resistant to TNF-induced shock and exhibit impaired upregulation of TNF-induced cytokines and chemokines in vitro and in vivo. By using bone marrow reconstitution experiments, we demonstrate that RIPK1 kinase activity in a non-hematopoietic lineage drives TNF-induced lethality. We establish that RIPK1 kinase activity is required for TNF-induced increases in intestinal and vascular permeability and clotting, and implicate endothelial cell necroptosis as an underlying factor contributing to TNF/zVAD-induced shock. Thus, work in this thesis reveals that RIPK1 kinase inhibitors may have promise in treating shock and sepsis.
# Table of Contents

Signature Page .................................................................................................................. ii

Acknowledgments ............................................................................................................. iii

Abstract ............................................................................................................................... iv

Table of Contents ................................................................................................................ v

List of Figures ...................................................................................................................... vi

CHAPTER I - Introduction .................................................................................................. 1

CHAPTER II – RIPK1 kinase activity mediates necroptotic complex formation and death in vitro ................................................................................................................. 45

   Introduction ...................................................................................................................... 46
   Results ............................................................................................................................... 48
   Discussion ......................................................................................................................... 55
   Methods ............................................................................................................................ 58

CHAPTER III - RIPK1 kinase inactive mice are resistant to TNF- but not LPS-induced shock ..................................................................................................................... 59

   Introduction ...................................................................................................................... 60
   Results ............................................................................................................................... 62
   Discussion ......................................................................................................................... 74
   Methods ............................................................................................................................ 77

CHAPTER IV – RIP kinase 1-dependent endothelial necroptosis underlies lethal TNF/zVAD-induced systemic inflammatory response syndrome ...... 79

   Introduction ...................................................................................................................... 80
   Results ............................................................................................................................... 82
   Discussion ......................................................................................................................... 108
   Methods ............................................................................................................................ 116

CHAPTER V - Discussion .................................................................................................... 124

References ............................................................................................................................ 141
List of Figures

Figure 1. Domain structure of RHIM-dependent signaling proteins.

Figure 2. Signaling pathway depicting TNF-induced regulation of NF-κB activation and apoptosis.

Figure 3. Signaling pathways depicting the mechanism and regulation of necroptosis induction and execution.

Figure 4. RIPK1, but not RIPK3, kinase activity is required in MEFs to mediate a stable RIPK1/RIPK3 interaction in response to a pro-necroptotic stimulus.

Figure 5. Efficient necrosome formation in response to TNF/Smac/zVAD stimulation in MEFs requires the kinase activity of RIPK1 but not RIPK3.

Figure 6. RIPK1 kinase activity is required for TNF/Smac-induced apoptosis and TNF/cycloheximide/zVAD- or TNF/Smac/zVAD-induced necroptosis in vitro.

Figure 7. RIPK1 kinase activity and RIPK3 mediate LPS/zVAD-induced necroptosis in primary macrophages in vitro.

Figure 8. Ripk1\textsuperscript{D138N/D138N} and RIPK3-deficient mice are protected from TNF- or TNF/zVAD-induced shock, a mouse model of systemic inflammatory response syndrome.

Figure 9. Ripk\textsuperscript{1D138N/+} littermate mice are protected from high-dose TNF/zVAD-induced shock due to gene dosage sensitivity or dominant negative kinase activity.

Figure 10. Ripk1\textsuperscript{D138N/D138N} non-littermate mice are partially resistant to low dose LPS-induced shock.

Figure 11. RIPK1 kinase inactive mice are susceptible to LPS-induced shock similar to their WT littermate controls.

Figure 12. Ripk1\textsuperscript{D138N/D138N} mice are impaired in their ability to upregulate an LPS-driven proinflammatory cytokine and chemokine signature in vivo.
Figure 13. RIPK1 kinase activity is required for LPS/zVAD-induced Tnf and Csf2 expression in primary macrophages in vitro.

Figure 14. Ripk1^{D138N/D138N} primary macrophages do not contribute to LPS or LPS/zVAD-induced TNF, IL-6, or IL-1β up-regulation in vitro.

Figure 15. Ripk1^{D138N/D138N} mice exhibit impaired cytokine and chemokine production in response to TNF and TNF/zVAD administration in vivo.

Figure 16. RIPK1 kinase activity contributes to TNF- and TNF/zVAD-induced CXCL1 and CXCL2 chemokine and IL-6 cytokine production in vitro.

Figure 17. Impaired recruitment of Ripk1^{D138N/D138N} neutrophils to the lung and liver in response to TNF or TNF/zVAD administration.

Figure 18. Non-hematopoietic RIPK1 kinase inactive cells mediate protection against lethal TNF- and TNF/zVAD-induced shock.

Figure 19. Kinase inactive RIPK1 bone marrow cells effectively repopulate lethally irradiated hosts.

Figure 20. Proinflammatory cytokines and inflammatory cell recruitment do not correlate with survival in a TNF-induced model of shock.

Figure 21. TNF/zVAD-treated RIPK1 kinase inactive hosts exhibit reduced cytokine and chemokine production compared to WT hosts reconstituted with RIPK1 kinase inactive hematopoietic cells.

Figure 22. Non-hematopoietic RIPK1 kinase inactive cells mediate protection against TNF-induced intestinal and vascular hyperpermeability and coagulation.

Figure 23. Kinase inactive RIPK1 mice are modestly protected from TNF-induced ileal but not colonic intestinal epithelial apoptosis.

Figure 24. Kinase inactive RIPK1 mice are protected from TNF- or TNF/zVAD-induced breaks in the liver endothelium and coagulation, and liver endothelial cell necroptosis induced by TNF/zVAD treatment.

Figure 25. RIPK1 kinase activity is required for lung endothelial necroptosis induced by TNF/Smac/zVAD treatment but not for upregulation of adhesion molecules in endothelial cells in vitro.
Figure 26. Kinase inactive RIPK1 mice upregulate adhesion molecules and chemokines but exhibit decreased P-selectin and Cxcl2 expression in lung and liver treated with TNF or TNF/zVAD.

Figure 27. Gating strategy and purity of endothelial cells isolated from TNF- or TNF/zVAD-treated WT and Ripk1D138N/D138N mice.

Figure 28. Phosphorylated MLKL is detected in liver endothelial cells from TNF/zVAD-treated but not TNF-treated mice, nor is cleaved caspase-3 detected in TNF-treated livers.
CHAPTER I

Introduction
The role of cell death in inflammation and disease

Cell death is an integral part of multicellular organism survival, playing important roles during development and maintaining tissue and organismal homeostasis by eliminating damaged or infected cells. However, deregulation or inhibition of cell death can lead to inflammation and disease. Thus, discovering the signaling pathways linking cell death and inflammation is crucial to understanding disease pathogenesis and potential therapeutic strategies. Over the last few hundred years, based on the study of cell morphology during various processes in organisms, an idea of the existence of regulated cell death and accidental cell death has emerged.

In 1972, Kerr and colleagues introduced ‘apoptosis’ to describe a type of morphologically distinct regulated cell death they had observed. Apoptotic morphology includes cell shrinkage, pyknosis (chromatin condensation), karyorrhexis (nuclear fragmentation), and apoptotic body formation. Subsequent studies, first in *C. elegans*, defined the genetic program of apoptotic death. Apoptosis in vertebrates was found to be mediated by a family of cysteine proteases termed caspases, since they cleave after aspartic acid residues. Caspases are synthesized as zymogens, and upon initiator caspase cleavage and activation, they activate executioner caspases like caspase-3 to cleave various cellular substrates and induce the morphological changes associated with apoptotic cell death. Two apoptotic pathways have been discovered: intrinsic and extrinsic apoptosis. The intrinsic pathway can be activated by signals such as
growth factor depletion, toxins, hypoxia or free radicals, and involves intricate regulation by anti- and pro-apoptotic Bcl2 family members, mitochondrial outer membrane permeabilization, release of second mitochondria-derived activator of caspases (SMAC) and cytochrome c from the mitochondria, and activation of the executioner caspase-9\textsuperscript{10}.

Meanwhile, the extrinsic pathway is induced by ligand binding to corresponding death receptors of the tumor necrosis factor (TNF) receptor gene superfamily (TNFRSF), such as Fas ligand binding to the Fas receptor or the cytokine TNF-\(\alpha\) binding to its receptor, TNF receptor 1 (TNFR1)\textsuperscript{11}. These death receptors were found to contain a roughly 80-amino acid long C-terminal “death domain” (DD)\textsuperscript{12,13} which allows for recruitment and interaction with cytoplasmic death-domain containing adaptor proteins such as TNF-receptor-associated death domain (TRADD)\textsuperscript{14}, receptor interacting protein kinase 1 (RIPK1)\textsuperscript{15}, and Fas-associated death domain (FADD)\textsuperscript{16,17}. Through its death effector domain (DED)\textsuperscript{18}, FADD binds the initiator caspase-8\textsuperscript{19,20}, which upon activation mediates downstream executioner caspase cleavage and induction of apoptosis.

Historically, apoptosis has been viewed as an immunologically silent, or tolerogenic type of cell death. Plasma membrane flipping and exposure of phosphatidylserine early during apoptosis allows for recognition and clearance of apoptotic cells and bodies by cells of the innate immune system such as resident tissue macrophages\textsuperscript{21,22}. Thus, apoptotic cells do not release their intracellular contents and their phagocytosis by innate immune cells typically doesn’t produce
an inflammatory response\textsuperscript{23}. While apoptosis occurring during development or normal cellular turnover certainly seems to be non-immunogenic, cell death due to infection or tissue injury with accompanying inflammation may be beneficial in alerting the immune system to repair the tissue or eliminate a pathogen. Indeed, excessive apoptosis in various mouse models, especially in epithelial barrier tissue such as the skin, can induce inflammation \textit{in vivo}\textsuperscript{24–27}, suggesting apoptosis can be inflammatory in certain contexts.

While intense study was devoted to regulated apoptotic cell death, a necrotic morphology was thought to be unregulated and accidental. Necrosis can be caused by harsh physical or chemical stimuli, such as trauma, burns, or rapid changes in temperature or pH. Morphologically, necrosis involves cytoplasmic and organelle swelling, plasma membrane breakdown, and release of damage-associated molecular patterns (DAMPs)\textsuperscript{2}. Over the last 30 years, many studies have shown that necrosis can be regulated, and multiple sub-types of programmed necrotic death have been delineated. In 1988, Laster et al., showed that depending on the cell line, TNF could induce apoptotic or necrotic cell death\textsuperscript{28}. In the late 1990s Peter Vandenabeele’s group and others demonstrated that, primarily in caspase-8 deficient cells or upon caspase inhibition, death receptor signaling downstream of TNF or Fas induces caspase-independent necrotic cell death\textsuperscript{29–35}. Importantly, Holler and colleagues found a requirement for the kinase activity of the death receptor signaling component RIPK1 in mediating Fas, TNF, or TNF-related apoptosis-inducing ligand (TRAIL)-induced caspase-8-independent
necrotic cell death\textsuperscript{36}. Chan et al. introduced the term “programmed necrosis” to refer to this RIPK1-dependent necrotic death and demonstrated in 2003 that TNF stimulation of vaccinia virus-infected Jurkat T cells induces programmed necrosis. Additionally, certain viral gene products can inhibit this death, suggesting a potential \textit{in vivo} role for programmed necrosis in regulating viral infection\textsuperscript{37}. Finally, in 2005 this regulated necrotic death was named “necroptosis”, and a small molecule inhibitor was identified that inhibits RIPK1 kinase activity\textsuperscript{38,39}. Research over the last decade has shown that death receptor-induced necroptosis, a regulated form of necrosis, is mediated by RIPK1\textsuperscript{24,39–41} and receptor interacting protein kinase 3 (RIPK3)\textsuperscript{41–44} kinase activity, leading to activation of the downstream pseudokinase mixed-lineage kinase like (MLKL)\textsuperscript{45–48}.

While necroptosis has been the most studied sub-type of regulated necrotic death, many other forms of programmed necrosis have been found, including pyroptosis, parthanatos, and ferroptosis. Parthanatos can be induced by DNA damage or reactive oxygen species (ROS) and involves overactivation of polyADP-ribose polymerase 1 (PARP1), the mitochondrial apoptosis inducing factor (AIF) and results in depletion of NAD+ and subsequently ATP\textsuperscript{49–52}. Ferroptosis, as the name suggests, involves iron-dependent ROS production and lipid peroxidation due to diminished glutathione levels and reduction in phospholipid glutathione peroxidase 4 (GPX4) enzyme activity\textsuperscript{2,53}. Lytic and inflammatory pyroptotic death can serve as a host defense mechanism as it is typically induced in immune system phagocytes, such as macrophages and
dendritic cells, in response to microbial infection\textsuperscript{54–56}. Pyroptosis can occur upon recognition of pathogen proteins or DAMPs and activation of canonical inflammasomes and caspase-1, or intracellular lipopolysaccharide (LPS) recognition by inflammatory caspase-11 in mice or caspase-4 and -5 in humans. Inflammatory caspase activation results in cleavage of the pyroptosis executioner, gasdermin-D, and caspase-1-mediated processing and release of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18\textsuperscript{54,55,57–60}.

Although there are some morphological differences between the various forms of regulated necrosis, they are thought to stimulate an inflammatory response due to plasma membrane leakage or rupture and exposure of intracellular contents (DAMPs) to the immune system. Thus, regulated necrosis is viewed as a more inflammatory and immunogenic death response in comparison to apoptosis. However, apoptotic cells may undergo secondary necrosis with accompanying plasma membrane leakage. This can occur if an apoptotic cell is not recognized and cleared, for example when excessive amounts of apoptosis overwhelm the phagocytosis response\textsuperscript{61,62}. A recent study even implicated limited DAMP release via membrane permeabilization during early apoptosis\textsuperscript{63}. On the other hand, necroptosis may dampen the inflammatory response in certain contexts by prematurely terminating the TNF- or LPS-driven pro-inflammatory cytokine and chemokine response\textsuperscript{64}. Additionally, DAMP release from necroptotic cells was not sufficient for efficient T-cell cross-priming and tumor immunity but
requires additional RIPK1-dependent NF-κB-induced transcriptional responses in the dying cells\textsuperscript{65}.

DAMPs constitute a variety of intracellular molecules including HMGB1, ATP, uric acid, mitochondrial DNA, heat shock proteins (HSPs), and IL-1\textsubscript{α}\textsuperscript{66,67}. Certain DAMPs, such as HMGB1 or ATP, are non-inflammatory and have important functions in cells but become immunogenic upon release from dying cells. Other DAMPs, such as IL-1\textsubscript{α} or IL-33, are alarmins, cytokine-like molecules that are typically stored in cells but can be secreted or released upon plasma membrane lysis\textsuperscript{67}. Inflammation is the body’s response to infection or tissue damage, acting to neutralize a pathogen or repair injury. While pathogens can be detected by innate immune cells via Toll-like receptors (TLRs) and other classes of pattern-recognition receptors (PRRs), various DAMPs may also be sensed by TLRs\textsuperscript{67,68}. Thus, DAMP release via cell death caused by trauma or other injury has the capacity to cause sterile inflammation by recruiting and activating innate immune cells like neutrophils and monocytes. These cells release pro-inflammatory cytokines and chemokines, such as TNF and IL-1, leading to activation of lymphocytes and adaptive immunity while also recruiting additional innate immune cells. In addition to cytokine production, neutrophils and macrophages secrete ROS and various proteases that can cause cell death and damage tissue\textsuperscript{66,68,69}. Therefore, DAMP release due to cell death and inflammation and immune cell activation may cooperate in feed-forward amplification loops and are thought to underlie the pathogenesis of many chronic inflammatory diseases.
Sterile inflammation has been implicated in atherosclerosis, cancer, autoimmune disease, Alzheimer’s, and ischemia/reperfusion injury\textsuperscript{68,69}. Deregulation of cell death also underlies many diseases. Insufficient or excessive apoptosis is implicated in cancer, neurodegenerative, cardiovascular, and autoimmune diseases\textsuperscript{10}, while secondary necrosis is linked to atherosclerosis, ischemia, and autoimmune disorders\textsuperscript{62}. DAMP release and necroptosis have been linked to cancer, shock and sepsis, ischemia/reperfusion injury, inflammatory bowel diseases (IBD) such as Crohn’s disease, and neurodegenerative diseases including Alzheimer’s\textsuperscript{70}. Two main pro-inflammatory cytokines, IL-1 and TNF, are also prominent in many diseases. IL-1 has a pathological role in rheumatoid arthritis, gout, cardiovascular diseases, type 2 diabetes, and hereditary autoinflammatory syndromes\textsuperscript{71}. TNF, meanwhile, contributes to the pathogenesis of IBD, rheumatoid arthritis, ankylosing spondylitis, and psoriasis\textsuperscript{72}.

The intricate and interconnected network of apoptotic and necrotic cell death, DAMP release, and inflammation underlying disease is exemplified by the fact that multiple regulators are shared between these pathways. One example is the kinase RIPK1, a prominent decision maker downstream of both cell survival and cell death signals. As the rest of this thesis will examine, RIPK1 helps determine whether and how a cell will die, thus dictating the nature of the immune response and contributing to various acute and chronic diseases.
RIPK1 kinase and TNF receptor 1 signaling

Overview of RIPK1 kinase structure and function

In 1995 RIPK1 was identified as the first member of what became a family of serine/threonine kinases termed receptor-interacting proteins\textsuperscript{15,73}. This family of 7 RIP kinases has distinct carboxy termini but shares significant homology in the N-terminal kinase domain and is related to members of the interleukin-1-receptor-associated kinase (IRAK) family involved in IL-1β and TLR signaling\textsuperscript{73,74}. Murine RIPK1 was identified in a yeast two-hybrid screen as a 74 kDa protein (656 amino acids) that could interact with the intracellular DD of the Fas and TNFR1 receptors and was constitutively expressed in most tissues. The N-terminal region had strong kinase homology, while the C-terminus contained a DD that allowed for death receptor (DR) interaction. Interestingly, transient overexpression of RIPK1 induced apoptosis, and deletion of the DD abrogated this response\textsuperscript{15}. RIPK1 was also found to interact with TRAIL-R1 (DR4) and TRAIL-R2 (DR5), death receptors for the cytotoxic ligand TRAIL\textsuperscript{75}. Mouse and human RIPK1 have 68% sequence homology, with the greatest homology found in the DD, illustrating the importance of this domain to RIPK1 function\textsuperscript{76}.

Subsequent studies demonstrated that RIPK1 could mediate both TNF-induced NF-κB activation\textsuperscript{76,77} and apoptosis\textsuperscript{76} by interacting with pro-survival and pro-death adaptor proteins TRADD\textsuperscript{76}, FADD\textsuperscript{78}, and TNF-receptor associated factor 2 (TRAF2)\textsuperscript{76}. RIPK1 interacts with FADD\textsuperscript{78} and TRADD via DD interactions, while both the kinase domain and intermediate domain (ID) can interact with TRAF2\textsuperscript{76}. 


Thus, the main functional domains of RIPK1 are the N-terminal kinase domain (aa 1-304), the ID (aa 304-553) and the C-terminal DD (aa 553-656)\textsuperscript{15,79} (Figure 1). RIPK1 was formally demonstrated to be a serine/threonine kinase that can autophosphorylate itself, but its kinase activity was not required for NF-κB activation since putting back a kinase-dead version into a RIPK1-deficient Jurkat T-cell line restored TNF-induced NF-κB signaling\textsuperscript{76,77}. RIPK1 is recruited to the TNFR1 in a TNF-dependent manner via DD interactions with TRADD, along with TRAF2\textsuperscript{76}. Overexpression and deletion analyses showed that the ID of RIPK1 was required for NF-κB activation while DD overexpression was necessary and sufficient for apoptosis induction but could act as a dominant negative inhibitor of TNF-induced NF-κB activation\textsuperscript{76,77}. Interestingly, caspase-8 cleaves RIPK1 at D324 during TNF-, Fas-, or TRAIL-mediated apoptosis. This cleavage suppresses NF-κB activation while increasing TNF-induced apoptosis through enhanced interaction with TRADD and FADD\textsuperscript{80}. Thus, cleavage of RIPK1 can shift cells from a pro-survival to a pro-death response downstream of DR signals.

The generation of RIPK1-deficient mice by Kelliher et al. helped clarify the function of RIPK1 in an \textit{in vivo} setting. Surprisingly, these mice were perinatal lethal, succumbing within 3 days of birth with signs of lymphoid and adipose tissue apoptosis, suggesting that RIPK1 can inhibit cell death \textit{in vivo}. Indeed, \textit{Ripk1}\textsuperscript{-/-} cells were sensitive to TNF-induced apoptosis but TNF-induced NF-κB activation was impaired. Meanwhile, RIPK1 was not required for Fas-induced apoptosis or TNF-induced JNK activation\textsuperscript{81}. While RIPK1 was not essential for TNF- or Fas-
Figure 1. Domain structure of RHIM-dependent signaling proteins. The kinases RIPK1 and RIPK3 interact through their RHIM domains with each other, and/or with the RHIM-domain containing innate immune adaptor proteins TRIF and ZBP1. RIPK1 contains an intermediate (ID) and death domain (DD) while TRIF contains a toll-IL-1 receptor (TIR) domain.
induced apoptosis\textsuperscript{81}, RIPK1 and its kinase activity were required for TNF-, Fas-, and TRAIL-induced necrotic death\textsuperscript{36}. Using \textit{Ripk1}\textsuperscript{-/-} mouse embryonic fibroblasts (MEFs), RIPK1 was shown to be necessary for TNF-induced activation of the mitogen-activated protein kinases (MAPKs) ERK, p38, and JNK\textsuperscript{82}. Interestingly, RIPK1 kinase activity was required for ERK but not Ikk, JNK, or p38 activation\textsuperscript{82,83}. Thus, these early studies began to unravel the complexity of RIPK1 kinase-dependent and kinase-independent effects on TNF-induced NF-κB and MAPK activation, apoptosis, and necrosis.

Similarly, the RIP kinase family member RIPK3 was originally shown to induce apoptosis and NF-κB activation upon overexpression of its C-terminus\textsuperscript{84,85}. While the N-terminal kinase domain shared homology with RIPK1, the C-terminus was unique and contained no DD motif. Indeed, RIPK3 was a serine/threonine kinase, contained autophosphorylation sites in its C-terminal domain and could auto-phosphorylate itself, but its catalytic activity was not required for NF-κB activation or apoptosis induction\textsuperscript{84–86}. RIPK3 interacted with RIPK1 via it’s C-terminus and was found to be recruited to TNFR1 upon TNF stimulation\textsuperscript{84,86}. However, while a third study confirmed that overexpression of the RIPK3 C-terminus could induce apoptosis, it inhibited RIPK1 and TNFR1-induced NF-κB activation\textsuperscript{86}. It was subsequently shown that both RIPK1 and RIPK3 contain a RIP homotypic interaction motif (RHIM), a critical 4-amino acid (I/V)Q(I/V)G sequence required for RIPK1/RIPK3 interaction (Figure 1). Interestingly, while RIPK1 phosphorylation of RIPK3 was not detected, RIPK3 could phosphorylate RIPK1,
leading to a reduction in TNF-induced NF-κB activation. Importantly, an intact RHIM was required for this RIPK3-mediated phosphorylation event and NF-κB inhibition. The generation of RIPK3 knock-out mice helped clarify some of these early discrepancies regarding the role of RIPK3 in NF-κB activation and apoptosis. Unlike RIPK1-deficient mice, Ripk3−/− mice are viable, indicating that RIPK3 is dispensable for normal murine development. Thymocytes from RIPK3-deficient mice are sensitive to various apoptotic stimuli while MEFs and primary bone marrow-derived macrophages (BMDMs) exhibit normal TNF- and LPS-induced NF-κB activation. Therefore, RIPK3 does not appear necessary for NF-κB activation and apoptosis, at least in MEFs and BMDMs, and this result helps show the importance of studying responses in physiological settings with genetic knock-out mice in addition to transient overexpression systems and cell lines in vitro.

While Ripk3−/− mice are viable, the perinatal lethality of RIPK1-deficient mice precluded the study of the physiological relevance of RIPK1 as a driver of both pro-survival and pro-death signals. To circumvent this issue and identify the lineages and cell types dependent on RIPK1 for survival, the Vandenabeele group and our group, in collaboration with Manolis Pasparakis, generated RIPK1 conditional knock-out mice. Upon Cre-mediated deletion of loxP sites flanking exon 3, a frame-shift generates a non-functional protein. RIPK1 also has kinase-dependent functions, especially with regards to initiating necroptotic death. The small molecule necrostatin-1 (Nec-1) was found to inhibit necroptosis by
acting as an allosteric type III inhibitor of RIPK1. Nec-1 and other necrostatins bind to a hydrophobic, allosteric back pocket of RIPK1 near the ATP-binding site, thus stabilizing RIPK1 in an inactive DLG-out conformation. While Nec-1 has poor metabolic stability in vivo, an optimized derivative 7-Cl-O-Nec1 (Nec1s) was developed with improved stability and pharmacokinetics. Additionally, the original Nec-1 molecule was found to function as a ferroptosis inhibitor through an unknown mechanism, have other off-target effects, and exhibit toxicity at lower, but not higher, concentrations in a murine model of TNF-induced lethal systemic inflammatory response syndrome (SIRS). Importantly, Nec-1 is actually the same molecule as methyl-thiohydantoin-tryptophan (MTH-Trp), an inhibitor of the immune regulator indoleamine 2,3-dioxygenase (IDO), whose expression can be increased by LPS or interferon gamma (IFNγ). IDO activity is increased in patients with severe sepsis and septic shock and is associated with mortality. Meanwhile, the optimized Nec-1s analog did not inhibit IDO or ferroptosis and was much more selective towards RIPK1 in comparison with the original Nec-1. However, primarily Nec-1 was used in murine models to address the role of RIPK1 kinase activity in inflammation and cell death. Thus, in addition to RIPK1 conditional knock-out mice, a kinase inactive knock-in mouse model of RIPK1 was necessary.

RIPK1 autophosphorylates multiple serine residues in its N-terminal kinase domain, including S161 in the activation segment (T-loop residues 156-196). The catalytic activity of many kinases is regulated by the phosphorylation status of...
the activation segment, and phosphorylation of S161 helps re-orient and open up the catalytic core of RIPK1\(^{39}\). Like most canonical kinases, RIPK1 retains the amino acids necessary for ATP binding and hydrolysis, including the catalytic triad (residues Lys45/Glu63/Asp156), the phosphate-binding loop (P-loop residues 24-31), and the catalytic loop (residues 136-143). K45 in the ATP binding pocket binds to the α- and β-phosphates of ATP while the P-loop residues are associated with β-phosphate binding, thus helping to anchor and orient the ATP. The key catalytic loop HKD motif (residues 136-138) contains the catalytic base aspartate that initiates transfer of the γ-phosphate to the substrate serine or threonine residue\(^{91,104}\). Multiple groups mutated these key residues to generate catalytically inactive RIPK1 mice. GlaxoSmithKline made a K45A knock-in point mutation in exon 3 while our group and Genentech targeted the catalytic loop aspartate in exon 4, generating \(\text{Ripk1}^{D138N/D138N}\) mice\(^{40,41,105}\). Recently, the P-loop residues G26 and F27 were deleted to create a third RIPK1 kinase-dead mouse\(^{106}\). Unlike RIPK1-deficient mice, all of these RIPK1 kinase inactive knock-in mice are viable\(^{40,41,81,105,106}\). The role of RIPK1 kinase activity \textit{in vitro} and \textit{in vivo} will be explored further in this thesis by utilizing the RIPK1 D138N mouse.

**Induction and regulation of TNF-induced NF-κB activation**

As introduced above, RIPK1 was found to mediate TNF-induced NF-κB activation, but its role in the process was unclear\(^{76,77,81}\). Research in the last 20 years has demonstrated the importance of ubiquitination in this signaling pathway. Ubiquitin is a 7kDa protein that can be covalently attached to substrate proteins as
a post-translational modification. Ubiquitination involves ATP-dependent ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Ubiquitin is conjugated to other ubiquitin molecules at an N-terminal methionine (M1) to form linear ubiquitin chains or at lysine residues (K6, K11, K27, K29, K33, K48, and K63), thus forming monoubiquitination or polyubiquitination linkages on substrate proteins and affording a high degree of signaling regulation. For instance, K48-linked chains typically target proteins for degradation, while K11, K63, and linear chains act as scaffolds for recruitment and assembly of various signaling complexes\textsuperscript{107}.

TNF binding initiates trimerization of the TNFR1 and assembly of signaling complex I, which includes the DD-containing adaptor proteins TRADD and RIPK1\textsuperscript{76,77}. TRADD recruits the E3 ubiquitin ligase TRAF2\textsuperscript{108}, and is a crucial scaffold protein as TRADD-deficiency inhibits TNF-induced NF-κB and MAPK activation and apoptosis in MEFs and partially reduces signaling in BMDMs\textsuperscript{109,110}. NF-κB subunits are sequestered in the cytoplasm in an inactive state by inhibitor of κB family member proteins (IκB), such as IκBα, which block the nuclear localization sequence (NLS). The heterodimeric IκB kinase (IKK) complex consists of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO), and upon TNF stimulation and activation of the IKK complex leads to IκB phosphorylation. This event results in K48-linked polyubiquitination and proteasomal degradation of the IκBs, allowing nuclear translocation of the NF-κB subunits and subsequent gene activation\textsuperscript{111}. Importantly, both TRAF2 and RIPK1
recruited the IKK complex to the TNFR1, and in a kinase-independent manner RIPK1 could interact with NEMO and mediate IKK activation, possibly via IKK oligomerization\textsuperscript{112–115}.

RIPK1 itself is polyubiquitinated in lipid rafts upon TNF stimulation\textsuperscript{116} by K48- and K63-linked chains, and TRAF2 was proposed as the E3 ligase that mediates K63-linked RIPK1 ubiquitination\textsuperscript{83,117}. RIPK1 ubiquitination may be required for recruitment of TGF-β activated kinase 1 (TAK1)\textsuperscript{83} that is thought to be the upstream regulatory kinase that phosphorylates and activates the IKK complex\textsuperscript{118–120}. Indeed, the adaptor proteins TAK1-binding protein 2 (TAB2) and TAK1-binding protein 3 (TAB3) preferentially bind to K63-linked polyubiquitin chains, including those on RIPK1, and mutations in the zinc finger binding domain abrogate TAK1 kinase and subsequent IKK complex activation and NF-κB signaling\textsuperscript{121,122}. Importantly, RIPK1 K63-linked polyubiquitination at lysine 377 (K377) was shown to be a critical ubiquitination site for TNF-induced NF-κB activation. NEMO could bind K63- but not K48-linked polyubiquitin chains\textsuperscript{123}, and a K377R mutation abolished RIPK1 polyubiquitination and could not recruit or activate TAK1 and NEMO/IKK complexes in a RIPK1-deficient Jurkat T-cell line\textsuperscript{124,125}. Thus, K63-linked polyubiquitinated RIPK1 may act as a scaffold to bind NEMO and TAB2/3 and bring TAK1 and the IKKs into close proximity for phosphorylation and activation. Additionally, K63-linked ubiquitination at RIPK1 K377 inhibits TNF-induced apoptosis through an early NF-κB-independent mechanism, and later in a NF-κB-dependent manner\textsuperscript{124–126}. 
Recent studies have helped to clarify the types of ubiquitin linkages involved in TNF-induced NF-κB activation and the E3 ligases responsible. TRAF2 can bind and recruit the E3 ligases cellular inhibitor of apoptosis 1 and 2 (cIAP1 and cIAP2) to the TNFR1 to ubiquitylate RIPK1 and activate NF-κB signaling\textsuperscript{127,128}. Small molecule IAP antagonists (Smac mimetics) induce auto-ubiquitination and proteasomal degradation of the cIAPs, resulting in TNF-induced apoptosis\textsuperscript{129,130}. Importantly, through genetic deletion or Smac mimetic treatment, both cIAP1 and cIAP2 were demonstrated to be critical for TNF-induced K63-linked RIPK1 ubiquitination and NF-κB activation and suppression of TNF-mediated apoptosis\textsuperscript{131–133}. cIAP1 was also found to promote K11-linked polyubiquitination of RIPK1, and NEMO could bind to these K11-linked chains\textsuperscript{134}. The linear ubiquitin chain assembly complex (LUBAC) mediates M1-linked linear ubiquitin chain formation on various TNFR1 signaling components including RIPK1 and especially NEMO. LUBAC consists of the adaptor proteins SHARPIN and HOIL-1, and the E3 ligase HOIP, and is recruited to various polyubiquitin chains conjugated by cIAP1/2 E3 ligase activity. LUBAC recruitment and linear ubiquitination is required for efficient IKK complex and NF-κB activation, and helps to inhibit apoptosis\textsuperscript{135–140}. Thus cIAP1/2 and LUBAC conjugate various polyubiquitin chains on RIPK1 and other TNFR1 complex members to help recruit and activate the TAK1 and IKK kinase complexes. While TAB2/3 exclusively bind to K63-linked chains, NEMO has the highest affinity for M1-linked linear chains, but can also bind to K11- and K63-linked polyubiquitin chains\textsuperscript{122,134,141,142} (Figure 2).
Figure 2. Signaling pathway depicting TNF-induced regulation of NF-κB activation and apoptosis. TNF-induced stimulation recruits TRADD, TRAF2, RIPK1, and cIAP1/2 to the TNFR1. E3 ligase activity by cIAP1/2 and the LUBAC complex adds K63- or linear (M1)-linked ubiquitin chains to RIPK1, thus recruiting and activating the TAK1/TAB2/TAB3 and NEMO/IKK complex, resulting in NF-κB and MAP kinase activation and pro-survival signaling. In the presence of translation inhibitors such as cycloheximide, a TRADD-dependent apoptotic complex IIa forms. If cIAPs, TAK1, or NEMO are inhibited or depleted, a RIPK1 kinase-dependent complex IIb forms, recruiting FADD and caspase-8 to mediate apoptosis.
Although initially discovered as a mediator of TNF-induced NF-κB activation, it seems that, depending on the cell type, loss of RIPK1 partially impairs TNF-induced signaling. Indeed, RIPK1-deficient MEFs, embryonic thymocytes and hepatocytes, and primary intestinal epithelial cells (IECs) are capable of activating NF-κB to varying extents in response to TNF.

It seems that in most cells RIPK1 may be required for full IKK and NF-κB activation, but in the absence of RIPK1, cIAP1/2 and LUBAC-mediated ubiquitination of other TNFR1 components can still generate a polyubiquitin scaffold necessary for delayed recruitment and activation of the IKK complex. Meanwhile, the generation of kinase inactive RIPK1 mice has confirmed that the kinase activity of RIPK1 is dispensable for TNF- or LPS-induced NF-κB and MAPK activation, at least in MEFs and BMDMs.

While E3 ligases and ubiquitination play a crucial role in regulating RIPK1 and TNF-induced signaling, a class of deubiquitinating enzymes (DUBs) that removes various ubiquitin chains helps to fine-tune the regulation of these pathways. CYLD is a DUB that removes K63- and M1-linked linear ubiquitin chains from RIPK1 and other TNFR1 signaling components, thus serving as a negative regulator of NF-κB signaling. CYLD is recruited to LUBAC via the HOIP-binding partner, SPATA2, and similar to loss of CYLD, SPATA2 downregulation or loss augments TNF-induced NF-κB and MAPK activation while suppressing apoptotic and necroptotic death induction. Meanwhile, the DUB A20 is upregulated in response to TNF stimulation, and can remove K63-linked
chains while adding K48-linked chains$^{117,154}$. Additionally, A20 can bind to linear ubiquitin chains and prevent their removal by CYLD$^{148,149}$. Mice deficient in A20 develop severe inflammation and have a shortened life-span, due to prolonged NF-κB activation and sensitivity to TNF-induced death$^{154}$. Thus, TNF signaling is intricately regulated to fine-tune the pro-inflammatory and pro-survival response, and suppress TNF-induced apoptosis.

**Role of RIPK1 in kinase-dependent and kinase-independent apoptosis**

TNF-induced apoptosis involves a cytoplasmic complex II formed sequentially after membrane-bound complex I dissociation$^{155,156}$. TRADD and RIPK1 associate with the DD adaptor FADD and the pro-apoptotic caspase-8 in complex II$^{155}$. The activity of complex II is typically inhibited by the catalytically inactive caspase-8 homolog cellular FLICE inhibitory protein (cFLIP), whose long (cFLIP$_L$) and short (cFLIP$_S$) isoforms can interact with caspase-8 to suppress apoptosis$^{157}$. cFLIP is a key NF-κB target gene, but cycloheximide (Cx) treatment rapidly decreases cFLIP expression levels and sensitizes cells to TNF-induced apoptosis$^{158,159}$. Thus, TNF-induced NF-κB activation (complex I) is a key cell survival checkpoint, but complex II mediated apoptosis inhibition is relieved by termination of NF-κB signaling, as can occur with protein synthesis inhibitors.

Interestingly, multiple complex II variants can form which are dependent or independent of RIPK1, but include at least FADD and caspase-8. Protein translation inhibition (Cx) combined with TNF stimulation results in the formation of a RIPK1-independent pro-apoptotic complex IIa which includes TRADD, FADD,
and caspase-8\textsuperscript{160}. However, a RIPK1-dependent apoptosis-inducing complex IIb is formed under conditions of TNF stimulation and cIAP loss, due to knock-down or Smac mimetic treatment\textsuperscript{131–133,160}. While cycloheximide reduces cFLIP but not cIAP1/2 levels, Smac mimetic treatment has the opposite effect. TNF/Smac-induced complex IIb formation and apoptosis requires RIPK1 deubiquitination, RIPK1 kinase activity and CYLD while TRADD and TRAF2 are not found in the complex\textsuperscript{160} (Figure 2). Thus, depending on the upstream signals, TNF can modulate TRADD or RIPK1-dependent apoptotic complex formation.

A separate cytoplasmic complex II initially discovered in tumor cell lines, termed the Ripoptosome, can form independently of death receptors in a RIPK1-kinase dependent manner upon IAP loss/inhibition due to Smac mimetic treatment, genotoxic stress (etoposide treatment), or TLR3 activation. This high molecular weight complex (2 MDa) contains RIPK1, FADD, Caspase-8, and can include RIPK3 and cFLIP. Depending on the stimulus and cell type, the Ripoptosome can induce caspase-8 mediated apoptotic or RIPK3-mediated necroptotic death, and both require RIPK1 kinase activity\textsuperscript{161,162}. In the absence of cIAPs, the presence and ratio of cFLIP\textsubscript{L} to cFLIP\textsubscript{S} isoforms regulates Ripoptosome assembly and the type of death induced\textsuperscript{162}. FADD-bound caspase-8 recruits the cFLIP isoforms to heterodimerize with pro-caspase-8 molecules\textsuperscript{163}. Interestingly, caspase-8 heterodimerizes with cFLIP\textsubscript{L}, but not cFLIP\textsubscript{S}, allowing for partial caspase-8 catalytic activity. While full proteolytic processing and apoptosis induction is inhibited, caspase-8 can still cleave certain substrates including RIPK1, RIPK3,
and CYLD to suppress necroptosis\textsuperscript{80,164–168}. Indeed, Ripoptosome formation and cell death was inhibited in cells with low RIPK1 or high cFLIP\textsubscript{L} levels, while cFLIP\textsubscript{S} suppressed Ripoptosome-induced apoptosis but promoted necroptosis due to lack of caspase-8 activity when complexed with the short FLIP isoform\textsuperscript{162}.

In addition to cIAP1/2 loss, TAK1 or IKKα/β kinase inhibition or loss of NEMO also promote RIPK1 kinase activity-dependent apoptosis via NF-κB-dependent and -independent mechanisms, both \textit{in vitro} and \textit{in vivo}\textsuperscript{169–173}. While TNF/Smac mediated apoptosis required RIPK1 deubiquitination and CYLD\textsuperscript{160}, TAK1 kinase inhibition induced complex IIb formation without affecting RIPK1 ubiquitylation, and RIPK1-kinase dependent apoptosis was independent of CYLD\textsuperscript{169}. Thus, recruitment of TAK1 or IKK complex members to the ubiquitin chains or the post-translational modifications mediated by these kinases on RIPK1, instead of the ubiquitin chains themselves, may sequester RIPK1 at the TNFR1 or by some other mechanism prevent complex IIb formation and RIPK1 kinase-dependent apoptosis. Interestingly, mice lacking the LUBAC component SHARPIN due to an inactivating mutation develop severe TNFR1-driven dermatitis and multi-organ inflammation, but all pathology is rescued by the RIPK1 kinase inactive K45A allele while caspase-8 heterozygosity or RIPK3/MLKL deletion rescues the dermatitis or partially suppresses the multi-organ inflammation, respectively\textsuperscript{26,27,105}. Thus, like cIAPs, LUBAC presumably keeps RIPK1 in a pro-survival state through its ubiquitination at the TNFR1.
The loss of RIPK1, and thus its scaffolding function, *in vitro* and *in vivo* provides compelling evidence for its pro-survival and anti-apoptotic roles in certain contexts. RIPK1-deficient or depleted MEFs or L929 cells, respectively, are sensitized to TNF-induced apoptosis\(^{81,174,175}\). TNF-stimulated RIPK1-deficient but not RIPK1 kinase inactive MEFs can activate apoptosis through the non-canonical NF-κB pathway due to degradation of TRAF2, cIAP1 and cFLIP\(_L\) levels. Thus, RIPK1 scaffolding properties stabilize these proteins and are important to sustain pro-survival signaling\(^{89,176,177}\). Similar to MEFs, RIPK1-deficient IECs and organoids express reduced amounts of cIAP1, TRAF2 and cFLIP and undergo rapid apoptosis *in vitro*\(^{89}\). RIPK1 suppresses caspase-8 dependent apoptosis and RIPK3-dependent necroptosis *in vivo* since RIPK1-deficient mice exhibit lymphoid and adipose tissue apoptosis and their lethality is rescued by combined deletion of caspase-8 (or FADD) and RIPK3\(^{178-180}\). Caspase-8 loss prevents apoptosis in the intestine, thymus, lung, and liver of RIPK1-deficient neonates but does not rescue the neonatal lethality\(^{180}\). Additionally, IEC-specific *Ripk1* knockout causes severe intestinal pathology and early lethality due to FADD/caspase-8-dependent IEC apoptosis\(^{89,90}\). Thus, RIPK1 serves pro-survival and pro-death roles downstream of TNF signaling, and can suppress or promote caspase-8-dependent apoptosis in a kinase-dependent and -independent manner.

**Mechanisms of necroptosis induction and execution**

TNF-induced necroptosis is mediated by RIPK1\(^{24,39-41}\) and RIPK3\(^{41-44}\) kinase activity dependent activation of the death executioner MLKL\(^{45-48}\). In addition
to suppressing apoptosis, components of the TNFR1 signaling pathway and complex II repress the induction of necroptosis. Indeed, cIAP1/2, TAK1, NEMO, TRAF2, and A20 have all been implicated as negative regulators of necroptosis in vitro, and in some cases in vivo. Necroptosis induction in vitro typically requires caspase inhibition, and catalytic activity of caspase-8:cFLIP heterodimers appears to suppress necroptotic activation, potentially by cleaving the kinases RIPK1 and RIPK3. However, the strongest evidence for complex II mediated repression of necroptosis comes from in vivo studies.

Genetic loss of Caspase-8, Fadd, or FLIP (cFlar gene) is lethal during mouse development around embryonic day 10-11 due to yolk sac vasculature defects. Conditional caspase-8 deletion in the endothelial compartment phenocopies caspase-8 loss, suggesting that endothelial cell death contributes to the compromised yolk sac vasculature. Interestingly, a catalytically inactive (C362A) but not a non-cleavable mutant (D387A) is embryonic lethal, indicating an important role for the catalytic activity but not apoptotic function of caspase-8 during embryogenesis. Early embryonic lethality is driven by TNFR1 signaling and RIPK1, as their deficiencies prolong survival to E16.5 or birth, respectively. Co-ablation of Ripk3 or Mlkl rescues Caspase-8/− or Fadd/− mice to adulthood, unequivocally demonstrating the importance of complex II in suppressing necroptosis in vivo during development. Additionally, FADD and caspase-8 also suppress RIPK3-mediated signaling to maintain adult homeostasis as conditional FADD or caspase-8 deletion in IECs or keratinocytes leads to...
inflammation, cell death and tissue disruption that is rescued by RIPK3 deficiency\textsuperscript{196–200}. Interestingly, given its role in suppressing both apoptosis and necroptosis, embryonic lethality of cFLIP deficiency is rescued only by concomitant loss of both \textit{Fadd} and \textit{Ripk3}\textsuperscript{168}. Thus, in most cases for necroptosis to ensue caspase-8 activity needs to be blocked.

A necroptotic complex IIc, or necrosome, is formed when TNF and cycloheximide or Smac mimetic treatment is combined with pharmacologic caspase inhibition by using zVAD-fmk (zVAD) or other pan-caspase inhibitors\textsuperscript{43,44}. Necrosome formation requires intact RHIM domains in RIPK1 and RIPK3, which allow for their stable interaction and may promote their oligomerization and the formation of amyloid-like fibril structures\textsuperscript{43,44,87,201}. RHIM-mediated interaction may result in conformational changes that allow for auto- and trans-phosphorylation between RIPK1 and RIPK3, thus stabilizing their interaction and activating the necrosome\textsuperscript{43,44}. RIPK3 was confirmed to be dispensable for TNF-induced NF-κB activation or apoptosis, but could phosphorylate RIPK1 \textit{in vitro}\textsuperscript{42–44,87,88}. Although in some cell types RIPK3 may be constitutively bound to FADD\textsuperscript{43,181}, RIPK3 was found to be recruited to complex II when zVAD was used to block caspase-8 activity\textsuperscript{43,44,160}. Even though RIPK1 could not mediate RIPK3 phosphorylation \textit{in vitro}, RIPK1 kinase activity is required for efficient necrosmome complex formation and necrosome-specific RIPK3 phosphorylation, as evidenced by Nec-1 treatment\textsuperscript{43,44,87}. Thus, kinase activity and intact RHIM domains of both RIPK1 and RIPK3 are crucial for their interaction, necrosome formation, and subsequent
necroptotic cell death\textsuperscript{43,44,87} (Figure 3). Since RIPK3 can be recruited to the apoptotic complex II or the Ripoptosome and mediate necroptosis if caspase-8 activity is suppressed, it is unclear if in certain cases the necrosome, forms independently\textsuperscript{43,44,161,162}. Instead, signaling context and activation or suppression of various complex II components, namely caspase-8, cFLIP, FADD, RIPK1, and RIPK3, may determine death outcome and transition from a pro-apoptotic complex IIb to a pro-necroptotic complex IIc/necrosome.

CYLD is a positive regulator of necroptosis, as knock-down or caspase-8-mediated CYLD cleavage suppresses RIPK3-dependent cell death, while mutating the caspase-8 cleavage site in CYLD promotes TNF-induced necroptosis\textsuperscript{160,166,181,202}. \textit{In vivo}, CYLD loss was able to partially suppress RIPK3-triggered inflammation and cell death in FADD-deficient IECs and keratinocytes\textsuperscript{196,197}. Interestingly, CYLD may not regulate RIPK1 ubiquitination in complex I but in the necrosome, as CYLD loss resulted in increased RIPK1 ubiquitination in the necrosome and impaired RIPK1 and RIPK3 phosphorylation. RIPK3 may also be polyubiquitinated in the necrosome, but CYLD deficiency attenuated TNF/zVAD- but not TNF/Cx/zVAD- or TNF/Smac/zVAD-induced necroptosis\textsuperscript{43,203}. Thus, CYLD activity supports necroptosis but is not essential for it to occur in all signaling contexts or cell types.

TNF-induced necroptosis can trigger downstream ROS\textsuperscript{181,204}, and RIPK3 was found to help activate metabolic enzymes and regulate TNF-induced ROS production\textsuperscript{42,44}. Indeed, it was thought that ROS production may be the mechanism
Figure 3. Signaling pathways depicting the mechanism and regulation of necroptosis induction and execution. Necroptosome formation occurs downstream of TNFR1, TLR3, and TLR4 receptors and the cytosolic innate immune sensor ZBP1 (DAI). In conditions of caspase inhibition (zVAD), RIPK1 and RIPK3 interact via their RHIM. Upon RIPK1 and RIPK3 interaction and kinase activation, the necroptosis executioner MLKL is recruited to RIPK3 and activated. TLR3 and TLR4 mediate necrosome formation via the RHIM-containing adaptor TRIF binding to RIPK3; the role of RIPK1 is context and cell-type dependent. When ZBP1 senses virus or dsDNA, it can recruit RIPK3 via its RHIM to mediate MLKL activation and necroptosis. RIPK1 blocks this ZBP1/RIPK3 interaction by an unknown mechanism.
of necroptosis execution. The mitochondrial phosphatase phosphoglycerate mutase family member 5 (PGAM5) and the mitochondrial fission factor dynamin-related protein 1 (Drp1) were reported to contribute to necroptosis as downstream effectors by causing mitochondrial fragmentation\textsuperscript{205}. However, numerous subsequent studies, both \textit{in vitro} and \textit{in vivo}, have demonstrated that PGAM5, Drp1, ROS, and even mitochondria themselves, are dispensable for necroptosis to ensue\textsuperscript{47,206–210}. Thus, cellular ROS may accompany necroptosis due to membrane rupture and/or cell stress, but is not required for its execution.

The pseudokinase MLKL, activated by RIPK3-mediated phosphorylation, is considered the sole effector necessary for necroptosis execution as demonstrated by the generation of \textit{Mlkl}\textsuperscript{−/−} mice\textsuperscript{47,48}. Initially identified in small molecule and shRNA screens, RIPK3 phosphorylates human MLKL on T357 and S358 and mutation of both sites blocks necroptosis but doesn’t affect necrosome formation\textsuperscript{45,46}. The small molecule necrosulfonamide (NSA) blocks necroptosis execution downstream of RIPK3 activation by targeting specifically human MLKL. Interestingly, there is a basal RIPK3/MLKL interaction in some cell types, but MLKL recruitment and increased binding is dependent on RIPK3 kinase activity and phosphorylation of human RIPK3 at S227\textsuperscript{46}. Similarly, TNF-induced phosphorylation of murine RIPK3 at T231 and S232 is essential for MLKL recruitment to the necrosome but not for initial necrosome assembly\textsuperscript{211,212}. Meanwhile, RIPK3-mediated MLKL phosphorylation, while crucial for necroptosis signaling, is dispensable for stable RIPK3/MLKL complex formation\textsuperscript{212}. 
Interestingly, mutations in the MLKL pseudoactive site result in constitutive, RIPK3-independent necroptosis$^{47}$.

MLKL consists of an N-terminal four-helix bundle domain connected to a C-terminal pseudokinase domain by a two-helix linker, or brace, region$^{47,212}$. The kinase-like domain lacks the P-loop and several other key residues, so even though it can bind ATP, MLKL is catalytically inactive$^{46,47}$. Phosphorylation of MLKL activation loop residues by RIPK3 results in a conformational shift which exposes the four-helical bundle domain of MLKL by alleviating its restraint by the C-terminal pseudokinase domain$^{47,212,213}$. MLKL oligomerizes and translocates to cellular membranes, where the 4-helix bundle domain acts as the necroptosis executioner by causing membrane permeabilization$^{213–218}$. Indeed, expression of the 4-helix bundle domain alone or with the two-helix linker results in constitutive necroptosis$^{213,216,218}$. However, the oligomeric state of MLKL, and whether MLKL mediates necroptosis by directly forming pores or through activation of ion channels remains a matter of on-going study. MLKL has been reported to form trimers, tetramers, and even octamers$^{215,216,219}$. One group found that MLKL interacts with a cation channel (TRPM7) to cause calcium influx, while another showed tetramerized MLKL translocates to lipid rafts and causes sodium influx$^{215,216}$. Recently, MLKL was demonstrated to form cation channels that are preferentially permeable to magnesium instead of calcium$^{220}$. Meanwhile, others have implicated a direct permeabilization mechanism by oligomerized MLKL binding to negatively charged membrane phosphatidylinositol
phosphates. Overall, RIPK3-mediated activation and phosphorylation of MLKL results in exposure of the MLKL 4-helical bundle domain and oligomerization and translocation to the plasma membrane where it mediates, directly or indirectly, pore formation and causes necroptotic death of the cell.

In certain in vitro contexts, necroptosis can actually proceed independently of RIPK1. In MEFs that overexpress RIPK3, TNF can, in a TRADD-dependent manner, mediate necroptosis in the absence of RIPK1. In L929 cells, TNF-induced necroptosis via RIPK3 occurs upon knock-down of caspase-8 and RIPK1. Similarly, in L929 cells and MEFs, RIPK1 knock-down was found to potentiate TNF-induced necroptosis when caspase-8 is inhibited with zVAD. Thus, at least in some contexts, RIPK1 may suppress RIPK3 activation by inhibiting RIPK3 activation and oligomerization. Three groups utilized artificial dimerization systems to assess the role of RIPK1 and RIPK3 oligomerization in necroptosis induction irrespective of upstream death receptor signaling. One study found that induced RIPK1 or RIPK3 dimerization could induce either apoptosis or necroptosis, although RIPK3-induced apoptosis didn’t require its kinase domain. This suggests that the mode of death is determined by the relative availability of caspase-8, FADD, and MLKL. Meanwhile, the other studies showed that the RIPK1/RIPK3 hetero-dimer recruits additional free RIPK3 into the amyloid scaffold to induce RIPK3 autophosphorylation and propagate the necroptotic signal, but RIPK1 can act to suppress spontaneous cytosolic RIPK3 activation by controlling its oligomerization.
Surprisingly, mice expressing a kinase dead RIPK3 D161N are embryonic lethal at E10.5, similar to mice deficient in complex IIb components. The mice succumb to massive apoptosis, and are rescued by RIPK1 or caspase-8 deficiency, although the apoptotic death in this context does not require RIPK1 kinase activity but rather scaffolding function to recruit FADD and caspase-8. Interestingly, three small-molecule inhibitors of RIPK3 kinase activity show concentration-dependent apoptosis sensitization by a similar RHIM-driven recruitment of RIPK1 and subsequent assembly of a caspase-8/FADD/FLIP complex. However, RIPK3\textsuperscript{K51A/K51A} kinase inactive mice are viable and healthy, suggesting that the D161N mutation or RIPK3 inhibitors induce a conformational change that exposes the RHIM for binding with RIPK1 to promote pro-apoptotic complex activation. Indeed, oligomerized RIPK3 induces apoptosis in MLKL-deficient cells. These studies portray an intricate regulatory network of complex II components and the decision to undergo apoptotic or necroptotic death, with multiple complex members exhibiting pleiotropic function.

**Post-translational modifications regulate RIP Kinase signaling**

Additional regulation of complex II members, especially RIPK1 and RIPK3, is mediated by post-translational modifications, namely phosphorylation and ubiquitination, that affect the stability of necrosome components. Heat shock protein 90 (HSP90) is a chaperone molecule that regulates the stability and function of various cellular proteins. RIPK1 is one such HSP90-associated protein since inhibition of HSP90 function results in proteasomal degradation of
RIPK1\textsuperscript{174,229}. RIPK3 also associates with the HSP90-CDC37 cochaperone complex, and this interaction is required for binding to RIPK1 and necrototic RIPK3 activation\textsuperscript{230}. Indeed, HSP90 inhibitors were protective in a rat model of TNF-induced SIRS. Interestingly the RIPK3/HSP90 interaction was observed in human and rat but not mouse cells\textsuperscript{230}. A reported mechanism of modulating RIPK3 stability is via the E3 ligase CHIP, which ubiquitylates RIPK3 and targets it for lysosomal degradation. CHIP deficiency increases RIPK3 expression levels and necroptosis sensitivity. \textit{Chip}\textsuperscript{-/-} mice die a few weeks after birth due to inflammation and cell death in the small intestine, but this phenotype is rescued by concomitant RIPK3 ablation\textsuperscript{231}. Thus, CHIP may be a negative regulator of necrosome formation and necroptotic death. HSP90 has also been reported to modulate MLKL stability, oligomerization, and membrane translocation, thus potentially acting on RIPK1, RIPK3, and MLKL to regulate necrosome stability and enhance TNF-induced necroptosis\textsuperscript{232–234}. MLKL may also be constitutively associated with TRAF2, while TNF stimulation and CYLD-dependent TRAF2 deubiquitination is required for their dissociation. Thus, TRAF2 potentially serves as a necroptosis suppressor \textit{in vitro} and \textit{in vivo}\textsuperscript{183}.

Phosphorylation of RIPK1 and RIPK3, mediated by several complex I components, helps fine-tune the TNF-induced response via positive and inhibitory phosphorylation events. Although mutating most individual serine residues in the RIPK1 kinase domain has minimal effect on RIPK1 kinase activity and TNF-induced necroptosis induction, a RIPK1 S89A mutant enhances both. Meanwhile,
a phosphomimetic RIPK3 S204D mutant caused necroptosis insensitive to Nec-1 inhibition. TAK1 transiently phosphorylates RIPK1 at S321 in response to TNF stimulation. This does not affect NF-κB activation, but blocking this phosphorylation promotes RIPK1 kinase-dependent apoptosis. Interestingly, sustained TAK1-mediated phosphorylation of multiple RIPK1 residues, including S321, promotes RIPK1 kinase-dependent necroptosis. The kinases IKKα and IKKβ phosphorylate RIPK1 to suppress RIPK1-mediated apoptosis and necroptosis downstream of TNF signaling in an NF-κB-independent manner. This phosphorylation occurs in complex I, and in vivo inhibition of the IKKs sensitizes mice to RIPK1 kinase-dependent death in a model of TNF-induced SIRS. However, the IKK complex does not seem to mediate RIPK1 S321 phosphorylation. Instead, this phosphorylation may be mediated by MAPKAP kinase-2 (MK2), a kinase induced downstream of TNF or LPS signaling by TAK1-mediated activation of p38, independently of the IKK complex. MK2 phosphorylates primarily cytosolic RIPK1 at S320/S321 and S335/S336 (human/mouse), and this inhibits RIPK1 kinase activation and helps prevent complex II formation and apoptosis/necroptosis when combined with inhibitory IKK-mediated phosphorylation of RIPK1 in complex I. Interestingly, it seems that a cytosolic RIPK1 pool contributes to complex II formation with complex I dissociated RIPK1. Similar to IKK inhibition, an MK2 inhibitor sensitizes mice to TNF-induced SIRS in a RIPK1 kinase-dependent manner. Finally, RIPK3 phosphorylation may be modulated by the phosphatase Ppm1b acting to limit
RIPK3 activation via T231 and S232 auto-phosphorylation, thus suppressing MLKL recruitment and necroptosis induction *in vitro* and *in vivo*. Ppm1b loss of function mice were sensitized to TNF-induced caecum damage and SIRS via RIPK3 activation\textsuperscript{240}.

Ubiquitination of various TNF signaling components also affects their activation status and helps regulate their function. For instance, RIPK1 is modified with linear and K63 ubiquitin chains in the necroosome, while RIPK3 contains K63 and K48-linked chains\textsuperscript{184,203,231,241,242}. RIPK3 is K63-ubiquitinated at lysine 5 (K5) in the necroosome, helping to support RIPK1/RIPK3 complex formation. This event is modulated by A20, as it deubiquitinates the RIPK3 K5 residue, thus negatively regulating necroosome formation and necroptosis. A20-deficient T cells and MEFs are susceptible to RIPK3-mediated necroptosis due to enhanced necroosome formation. Indeed, RIPK3 deletion rescues the inflammation and significantly prolongs the survival of A20\textsuperscript{-/-} mice\textsuperscript{184}. However, while a separate study demonstrated that RIPK3 deficiency or catalytically inactive RIPK1 delay lethality of A20-deficient mice, MLKL deficiency had no protective effect\textsuperscript{227}. Thus, RIPK3-dependent signaling but not necroptosis contributes to the lethality of A20-deficient mice. Meanwhile, K63-linked RIPK1 ubiquitination in the necroosome is not mediated by cIAP1/2, but M1-linked ubiquitination seems to be mediated by LUBAC\textsuperscript{241}. RIPK1 ubiquitination in the necroosome also requires RIPK1 kinase activity, while RIPK3 and MLKL are dispensable\textsuperscript{242}. Mutating a necroptosis-associated ubiquitination site (K115R) reduced RIPK1 ubiquitination and
phosphorylation, thus disrupting necrosome assembly and induction of necroptosis. This suggests that RIPK1 ubiquitination in the necrosome contributes to maintaining RIPK1 kinase activation and necrosome formation. The K115R mutation had no effect on RIPK1 ubiquitination at complex I or TNF-induced NF-κB activation\textsuperscript{242}. Interestingly, a recent study implicated the E3 ligase Pellino 1 (PELI1) in mediating K63-linked ubiquitination of RIPK1 at K115 during necroptosis. This ubiquitination also required RIPK1 kinase activity and PELI1 promoted RIPK1 activation and necrosome assembly\textsuperscript{243}. Thus, coordinated RIPK1 phosphorylation and ubiquitination in the necrosome seems to contribute to the regulation of necroptosis signaling. While tremendous progress has been made, these studies convey the complexity of regulation and pleiotropy of TNFR1 signaling components, and raise more questions regarding how cells decide to undergo apoptosis or necroptosis.

**Toll-like receptors and necroptosis**

**Toll-like receptor signaling and the RIP kinases in innate immunity**

The TLR family of PRRs play an important role in innate immunity, as the TLRs are critical for sensing and responding to various pathogens and pathogen-associated molecular patterns (PAMPs) outside the cell or in endosomes. TLRs have an extracellular leucine-rich repeat (LRR) segment which allows for distinct ligand recognition. TLRs involved in recognition of microbial components (such as TLR2, TLR4, and TLR6) are located at the plasma membrane. Meanwhile, TLRs involved in nucleic acid recognition (e.g. TLR3, TLR7, and TLR9) are localized to
the endosomal membrane. The TLRs contain a cytoplasmic Toll/IL-1R (TIR) homology domain which recruits TIR domain-containing adaptors to propagate downstream signaling cascades activating NF-κB, MAPK, and interferon (IFN) pathways. Myeloid differentiation primary response 88 (MyD88) is the most universal of the 5 TIR adaptors, mediating signaling downstream of various TLRs except for TLR3.

TLR3 recognizes virus-derived or synthetic poly(I:C) double-stranded RNA (dsRNA) while TLR4 is activated upon LPS binding. Stimulation of these two receptors activates NF-κB, MAPK, and IFN responses via MyD88-independent pathways through TIR-domain-containing adaptor-inducing IFN-β (TRIF). TRIF is directly recruited to TLR3 but associates with TLR4 through the TRIF-related adaptor molecule (TRAM). Analysis of TRIF-deficient mice demonstrated that TLR3/4-induced interferon regulatory factor 3 (IRF3) activation and downstream IFN signaling requires TRIF while TLR-induced NF-κB activation can proceed downstream of TLR4 but not TLR3 via the MyD88-dependent pathway. Interestingly, the C-terminus of TRIF contains a RHIM, allowing it to bind to RIPK1 and RIPK3 (Figure 1). RIPK1 mediated the TRIF-dependent NF-κB activation induced by TLR3 and TLR4 but was dispensable for IRF3 activation and the IFN response. RIPK1 was also phosphorylated and ubiquitinated in TLR3-stimulated cells, while RIPK3 inhibited TRIF-induced NF-κB activation by competing for binding to RIPK1. Thus, TRIF recruits RIPK1 via RHIM interaction to activate NF-κB signaling downstream of TLR3 or TLR4.
The cytoplasmic DNA sensor Z-DNA binding protein 1 (ZBP1), also known as DAI (DNA-dependent activator of IFN-regulatory factors), is an IFN-inducible sensor that can bind dsDNA and activate IFN and NF-κB signaling\textsuperscript{258,259}. Interestingly, ZBP1 contains two RHIM sequences, and recruits both RIPK1 and RIPK3 to activate NF-κB in a RHIM-dependent manner\textsuperscript{259,260} (Figure 1). This ZBP1-induced signaling is disrupted by the murine cytomegalovirus (MCMV) M45-encoded viral inhibitor of RIP activation (vIRA), which contains a RHIM and acts to suppress RIPK1-dependent TNF-induced signaling and necroptosis during infection\textsuperscript{261,262}. Thus, viral proteins can inhibit pro-inflammatory and cell death signaling via RHIM-dependent interactions with host proteins RIPK1 and RIPK3.

**Other stimuli that induce necroptosis**

TRIF and ZBP1 interaction with RIPK1 and/or RIPK3 via RHIM can mediate necroptosis instead of proinflammatory signaling when caspase function is inhibited. For instance, during MCMV infection ZBP1 interacts with RIPK3 via RHIM and activates MLKL to mediate virus-induced necroptosis in a RIPK1-independent manner while the RHIM-containing MCMV vIRA suppresses necroptosis induction by interfering with ZBP1/RIPK3 complex formation\textsuperscript{263,264}. Surprisingly, genetic evidence demonstrated that ZBP1 drives RIPK3/MLKL-mediated necroptosis in \textit{Ripk1\textsuperscript{-/-}} mice, indicating that RIPK1 maintains developmental homeostasis by acting via its RHIM to suppress ZBP1-mediated activation of RIPK3\textsuperscript{265,266}. Thus, ZBP1-driven necroptosis requires RIPK3 and MLKL but proceeds independently of RIPK1.
If caspases are inactivated, TRIF mediates necroptosis via RHIM-dependent RIPK1/RIPK3 recruitment downstream of poly(I:C) or LPS stimulation of TLR3 and TLR4. RIPK1 kinase activity seems to be required for necroptosis in macrophages but is dispensable for TLR3-induced necroptosis in fibroblast and endothelial cell lines\textsuperscript{267,268}. Type I (IFNα/IFNβ) or type II (IFNγ) interferons induce necroptosis in FADD-deficient or caspase-inhibited MEFs in a RIPK1- and RIPK3-dependent manner. Interferons transcriptionally upregulate the RNA-responsive protein kinase R (PKR) in MEFs to interact with RIPK1 and mediate necrosome formation\textsuperscript{178,269}. Surprisingly, RIPK1-deficient primary MEFs are sensitized to poly(I:C) or interferon-mediated RIPK3-dependent necroptosis without the need for caspase inhibition, and TRIF or IFNαR ablation prolongs survival of Ripk1\textsuperscript{-/-}/Tnfr1\textsuperscript{-/-} mice\textsuperscript{178}, revealing that RIPK1 can potentiate or repress TLR and IFN-induced necroptosis. In macrophages however, LPS, poly(I:C), or TNF-induced necroptosis in the presence of caspase inhibitors requires type I IFN signaling but PKR is dispensable\textsuperscript{270}. A type I IFN-induced IRF9/STAT1/STAT2 complex leads to sustained RIPK1 and RIPK3 phosphorylation and necrosome activation, thus promoting TLR/TRIF- or TNF-induced necroptosis\textsuperscript{270}. MLKL appears to be an interferon-stimulated gene\textsuperscript{178,269,271}, thus clarifying how interferons prime macrophages and increase their sensitivity to LPS- and TNF-induced necroptosis. Macrophage necroptosis, therefore, incorporates interferon, TLR/TRIF, and TNF-induced signaling to mediate death.
Cross-talk between inflammation and cell death in mediating disease

RIPK1 death-independent roles in pro-inflammatory signaling

In addition to its role in mediating NF-κB activation or cell death in response to TNF stimulation, additional RIPK1 death-independent effects on cytokine/chemokine production have recently emerged. In LPS-stimulated bone marrow-derived dendritic cells (BMDCs), RIPK3 assembles a complex with FADD, caspase-8, and RIPK1 to mediate pro-IL-1β processing. This effect does not depend on the kinase activities of RIPK1 or RIPK3. In caspase-8-deficient DCs, RIPK1 kinase activity but not RIPK3 drives systemic autoimmunity by hyper-responsive TLR4/7/9-induced proinflammatory cytokine secretion. Interestingly, a separate study reported RIPK1 kinase activity, RIPK3, and MLKL-dependent LPS-induced inflammasome activation and IL-1β secretion in caspase-8-deficient DCs, occurring independently of necroptosis. cIAP loss in macrophages activates RIPK1 kinase-dependent TNF transcription and RIPK3-dependent TNF secretion in an MLKL-independent manner, leading to enhanced proinflammatory cytokine production. Indeed, RIPK1 kinase activity in several cell lines, including L929 cells, was shown to regulate TNF production upon caspase inhibition, though a potential role for RIPK3 in this context has not been addressed. RIPK1 has also been reported to regulate TNF/zVAD or LPS/zVAD-induced cytokine responses in BMDMs in vitro and in vivo, in a kinase-dependent but necroptosis-independent manner. Furthermore, IL-1α production and autoinflammation in a SHP-1 mutant mouse (Ptpn6<sup>spin</sup>) is mediated by RIPK1 kinase independently
of RIPK3\textsuperscript{279}. Depending on the context and cell type, RIPK1 and/or RIPK3 can mediate inflammatory signaling in a kinase-dependent or kinase-independent manner. Thus, the pleiotropy of RIPK1 makes it challenging to unravel the contribution of RIPK1 scaffolding or kinase function to inflammatory or cell death processes in the context of infection or disease.

**Necroptosis in disease models**

RIPK1- and RIPK3-driven inflammation and/or necroptosis have been implicated in various mouse models of disease or infection, but only recently have we begun to discern differences between inflammatory or cell death contribution through the use of MLKL-deficient mice. Many viruses encode caspase inhibitors, so necroptosis can act as a host defense mechanism in case apoptosis is suppressed. Indeed, RIPK3-induced necroptosis in Vaccinia virus-infected cells suppresses inflammation and helps control infection in vivo\textsuperscript{43}. Similarly, MCMV-encoded vIra targets DAI and RIPK3 via RHIM to disrupt their interaction and suppress virus-induced necroptosis\textsuperscript{263,264}. Herpes simplex viruses (HSV) HSV-1 and HSV-2 encode RHIM-containing proteins that are bound by RIPK1 and RIPK3 to induce necroptosis and restrict viral propagation in mice\textsuperscript{280}. Interestingly, these HSV-encoded proteins suppress necroptosis induction in human cells\textsuperscript{280,281}. Meanwhile, infection with Influenza A virus (IAV) results in activation of both apoptosis and necroptosis to limit viral replication upon sensing by ZBP1. ZBP1 activation of RIPK3 mediates both RIPK1/FADD/caspase-8-dependent apoptosis and RIPK3/MLKL-dependent necroptosis in infected cells\textsuperscript{282–284}. 
Bacteria can also modulate the type of cell death induced during infection. RIPK1, RIPK3 and caspase-8 in macrophages activate NF-κB and caspase-1-mediated inflammasome signaling and induce apoptotic and pyroptotic cell death to help control *Yersinia pestis* infection and regulate host resistance\textsuperscript{285,286}. If caspases are inhibited, RIPK1 and RIPK3 mediate necroptotic death of infected cells\textsuperscript{285,286}. Interestingly, enteropathogenic *Escherichia coli* (EPEC) secretes the cysteine protease EspL during infection to cleave all RHIM-containing host proteins, thus inhibiting host-mediated inflammatory and necroptotic responses\textsuperscript{287}. Infection with *Salmonella enterica serovar Typhimurium* induces cell death by type I IFN-driven RIPK1/RIPK3-mediated macrophage necroptosis although RIPK3-deficiency did not provide a survival advantage *in vivo*\textsuperscript{288}. Thus, depending on the cause of infection, viral or bacterial-induced necroptosis can be detrimental to the host or serve as a defense mechanism.

Although genetic evidence supports the role of RIPK1 in suppressing caspase-8-mediated apoptosis and RIPK3/MLKL-mediated necroptosis\textsuperscript{178–180}, RIPK1 and RIPK3 have been implicated in mediating inflammation and necroptosis in various diseases including shock, ischemia-reperfusion, and neurodegenerative diseases\textsuperscript{289,290}. RIPK3, MLKL, and RIPK1 kinase inactivity (by Nec-1 inhibition) have been shown to drive TNF-induced hypothermia, shock, and lethality, thus implicating necroptosis in shock and sepsis\textsuperscript{97,227}. Nec-1 was originally shown to be protective in a model of ischemia-reperfusion injury in the brain\textsuperscript{38}. Subsequently, various studies have implicated RIPK1 kinase activity and RIPK3 as protective in
models of heart and kidney ischemia-reperfusion injury\textsuperscript{291–293}. However, MLKL-deficient mice were recently shown to be less resistant to kidney ischemia-reperfusion injury, thus raising questions regarding the overall contribution of necroptosis to this model\textsuperscript{227}. Interestingly, necroptosis may play an important part in neurodegeneration, as RIPK1, RIPK3, and MLKL expression and activation is elevated in pathological samples of patients with multiple sclerosis or ALS\textsuperscript{294,295}. Thus, while RIPK1 contributes to various diseases, it will be important to discern between the contribution of inflammation and cell death in future studies of these disease models.

An open question in the cell death field is the physiological relevance of necroptosis in normal biology. As discussed above, RIPK1/RIPK3-mediated inflammation and MLKL-dependent necroptosis are being actively studied and implicated in various pathophysiological conditions of mouse and human infection and disease. However, does necroptosis have a role during development or organismal homeostasis, like caspase-8 does with organ and body formation and regulation of cell populations? The viability of RIPK1 kinase inactive, \textit{Ripk3}\textsuperscript{−/−} and \textit{Mlkl}\textsuperscript{−/−} mice suggests that necroptosis is dispensable for murine development and normal homeostasis under non-challenged conditions\textsuperscript{40,41,47,48,88,105}. Indeed, necroptosis is typically observed \textit{in vitro} or in mouse models \textit{in vivo} only under conditions of inhibition or deletion of the upstream negative regulators like cIAP1/2, FADD, caspase-8, TAK1, or NEMO. Thus, current data argue that necroptosis is actively suppressed during development and under steady state conditions, but
improved biomarkers and additional experimental techniques will be needed to fully address whether necroptotic death has a physiological role during development or organismal homeostasis.

In this thesis, I focus on defining and elucidating the role of RIPK1 kinase activity in mediating inflammatory and death responses. Specifically, in chapters 2 and 3 I address the role of RIPK1 kinase activity in TNF- or LPS-induced transcriptional and translational cytokine/chemokine responses and the induction of necroptotic complex formation and death in vitro. Utilizing RIPK1 kinase inactive mice, I elucidate the contribution of RIPK1 kinase activity to inflammatory and cell death processes in a mouse model of TNF-induced shock in chapters 3 and 4.
CHAPTER II

RIPK1 kinase activity mediates necroptotic complex formation and death in vitro


*All experiments and data analysis in Figures 4 to 7 were performed by Matija Zelic
**Introduction**

RIPK1 plays a prominent role in proinflammatory and cell survival signaling by mediating optimal NF-κB and MAP kinase pathway activation downstream of TNFR1 and TRIF-dependent TLRs. In addition to pro-survival signaling, RIPK1 can induce caspase-8 dependent apoptosis or caspase-independent necroptosis. Under conditions of caspase inhibition, the related RIPK1 and RIPK3 kinases interact through their RHIMs, resulting in auto- and trans-phosphorylation and RIPK3-mediated phosphorylation of the downstream pseudokinase MLKL. Phosphorylation of MLKL results in its oligomerization and translocation to the plasma membrane. Activated MLKL mediates membrane permeabilization and necroptosis, leading to the release of DAMPs. Necroptosis requires the kinase activities of RIPK1 and RIPK3 and can be activated downstream of death receptors (TNF, FAS, TRAIL), TLRs 3 and 4, type I or II interferon receptors (IFNαR or IFNγR) and the cytosolic viral sensor ZBP1^{289,290}.

Although reported to be dispensable for TNF/Cx-induced apoptosis, TNF/Smac-induced complex IIb formation and apoptosis induction was shown to depend on RIPK1 kinase activity in HT29 cells^{160}. Meanwhile, necrosome (complex IIc) formation required intact RIPK1 and RIPK3 RHIM domains and kinase activity in cells treated with TNF and zVAD combined with either cycloheximide or Smac mimetics^{43,44}. Since RIPK1 kinase-dependent effects were interrogated with *in vitro* reconstitution studies or using the small molecule inhibitor of RIPK1 kinase activity, Nec-1^{38,39}, interpretation of these studies is limited by
compound instability and off-target effects\textsuperscript{99}. Additionally, necroptosis can proceed independently of RIPK1 in certain cell types, as RHIM-containing ZBP1 or TLR3/4 adaptor TRIF can directly interact with RIPK3\textsuperscript{264,267,268}. To determine the contribution of RIPK1 kinase activity to development and tissue homeostasis \textit{in vivo}, we generated knock-in mice expressing a kinase-inactive mutant RIPK1 from the endogenous \textit{Ripk1} locus. We replaced the conserved aspartate (D) at position 138 within the activation loop of the RIPK1 kinase domain with asparagine (\textit{Ripk1\textsuperscript{D138N}}), thus rendering the mice catalytically inactive\textsuperscript{40}. Unlike RIPK1-deficient mice, and similar to RIPK3 and MLKL-deficient mice, these RIPK1 D138N knock-in mice were viable and allowed us to re-assess the role of RIPK1 kinase activity in complex II formation and cell death regulation\textsuperscript{40,47,48,81,88}. Utilizing MEFs and primary bone marrow-derived macrophages from these mice, we demonstrate the requirement for RIPK1 kinase activity in necrosome formation and in TNF- or LPS-induced necroptosis.
Results

RIPK1 but not RIPK3 kinase activity is required for necroptotic complex formation in MEFs

The kinase activities of both RIPK1 and RIPK3 have been implicated as necessary for stable necrosome complex formation and induction of necroptotic death\(^{43,44}\). To address the role of RIPK1 kinase activity using endogenous kinase inactive protein instead of chemical inhibitors, we immortalized MEFs from WT, \textit{Ripk}^{1\text{D138N/D138N}}\textit{1}, and \textit{Ripk}^{3\text{-/-}} mice. We found that RIPK3 and the kinase activity of RIPK1 are required for stable RIPK1/RIPK3 interaction and necrosome formation upon TNF/Smac/zVAD stimulation (Figure 4A). Complex formation was rapid, as we could detect a RIPK1-RIPK3 interaction as early as 1-2 hours after TNF/Smac/zVAD stimulation. As expected, TNF/Smac treatment, a pro-apoptotic stimulus, did not induce a RIPK1/RIPK3 interaction. Interestingly, inhibition of RIPK3 kinase activity with GSK843A or GSK872B seemed to potentiate RIPK1/RIPK3 interaction and necrosome formation (Figure 4B) while addition of Nec-1 or GSK963A, two RIPK1 kinase inhibitors, blocked RIPK1/RIPK3 interaction. Perhaps GSK843A and GSK872B stabilize RIPK3 in a kinase inactive formation with RIPK1, thus blocking MLKL recruitment and activation, thereby sustaining the RIPK1/RIPK3 interaction. Indeed, RIPK1 in the whole cell lysate migrates as two bands only in instances of RIPK1/RIPK3 complex formation, with the slower migrating band typically indicative of phosphorylation (Figure 4). Although we did not test this hypothesis directly with the addition of phosphatase,
RIPK1 may be phosphorylated upon TNF/Smac/zVAD treatment in the absence of RIPK3 kinase activity (Figure 4).

While we could not detect caspase-8 above the Ig heavy chain band in this complex (Figure 4A), immunoprecipitating caspase-8 in TNF/Smac/zVAD-treated cells revealed a RIPK1/RIPK3/caspase-8 interaction that required RIPK1 but not RIPK3 kinase activity for complex formation (Figure 5A). Protein levels seemed to decrease in the whole cell lysate upon complex formation, suggestive of necrosome aggregation into a buffer-insoluble fraction (Figure 5A). Interestingly, the N-terminal FLIP$_L$ fragment FLIP(p43), processed by caspase-8, was increased upon stable complex formation (Figure 5B). FLIP(p43) blocks pro-caspase-8 cleavage but unlike FLIP$_L$ allows for caspase-8/RIPK1 interaction$^{296,297}$. Overall, our results indicate that the kinase activity of RIPK1, but not RIPK3, is required in MEFs for stable necrosome formation and RIPK1/RIPK3 interaction upon treatment with the pro-necroptotic TNF/Smac/zVAD stimulus.
Figure 4. RIPK1, but not RIPK3, kinase activity is required in MEFs to mediate a stable RIPK1/RIPK3 interaction in response to a pro-necroptotic stimulus. WT, Ripk1<sup>D138N/D138N</sup> and Ripk3<sup>−/−</sup> SV40-immortalized MEFs were pre-treated with Smac and/or zVAD for 1 hour (A) or additional RIPK1 and RIPK3 kinase inhibitors (B), before treating with TNF for 2 hours. A representative western blot showing protein lysates from RIPK3 immunoprecipitates or whole cell lysate probed with antibodies to RIPK1, RIPK3, or caspase-8 is depicted.
Figure 5. Efficient necrosome formation in response to TNF/Smac/zVAD stimulation in MEFs requires the kinase activity of RIPK1 but not RIPK3. WT, Ripk1<sup>D138N/D138N</sup> and Ripk3<sup>−/−</sup> SV40-immortalized MEFs were pre-treated with Smac, zVAD, and RIPK1 and RIPK3 kinase inhibitors for 1 hour (A) prior to treatment with TNF for 2 hours. Representative western blots depict protein lysates from caspase-8 immunoprecipitates or whole cell lysate probed with antibodies to RIPK1, RIPK3, and caspase-8 (A), or cFLIP and ERK1/2 in the whole cell lysate (B).
**RIPK1 kinase activity is required for TNF/Smac-induced apoptosis and TNF/Cx/zVAD- or TNF/Smac/zVAD-induced necroptosis**

While reported to be dispensable for TNF/Cx-induced apoptosis, the kinase activity of RIPK1 was implicated in both TNF-induced apoptosis and necroptosis under conditions of cIAP depletion and/or caspase inhibition\(^{44,160}\). In agreement with these studies, we found that RIPK1 kinase activity in MEFs is not necessary for TNF/Cx-induced apoptosis but is required for apoptosis induced by TNF and Smac mimetic treatment or necroptosis induced by either TNF/Cx/zVAD or TNF/Smac/zVAD stimulation (Figure 6). As expected, RIPK1-deficient MEFs were sensitized to TNF/Cx-induced apoptosis\(^8\) but resistant to TNF/Cx/zVAD-induced necroptosis, given the requirement for RIPK1 kinase activity (Figure 6A). RIPK1 kinase inhibition by small molecule inhibitors Nec-1 or GSK963A rendered MEFs resistant to necroptotic stimuli (Figure 6). Interestingly, RIPK3 kinase inhibitors also blocked TNF/Smac/zVAD-induced necroptosis even though necrosome formation was not impaired in these cells (Figure 5 and 6B). Thus, necrosome formation requires RIPK1 kinase activity but necroptosis execution requires the kinase activities of both RIPK1 and RIPK3 in this setting.
Figure 6. RIPK1 kinase activity is required for TNF/Smac-induced apoptosis and TNF/cycloheximide/zVAD- or TNF/Smac/zVAD-induced necroptosis in vitro. SV40-immortalized MEFs generated from WT, Ripk1D138N/D138N, and Ripk1-/- mice were treated with TNF (10 ng/ml), zVAD (20 μM), Smac mimetic (1 μM), cycloheximide (1 μg/mL), Nec-1 (30 μM), and GSK963A/GSK843A/GSK872B (3 μM) for 20 hours and cell viability was measured using MTS assay (n = 3 biological replicates). Error bars represent SEM.

RIPK1 kinase activity mediates necroptosis upon LPS/zVAD treatment in primary macrophages

The TLR3/4-specific adaptor TRIF recruits RIPK1 via the RHIM to mediate NF-κB activation; when apoptosis is inhibited, it is thought to recruit RIPK1 and RIPK3 to mediate necroptosis. However, TRIF-dependent necroptosis can occur in the absence of RIPK1 and may be mediated by the direct recruitment of RIPK3 to TRIF and TLR3/4. To determine whether the kinase activity of RIPK1 mediates TRIF-dependent necroptosis, primary BMDMs from wild-type (WT) or
Ripk1D138N/D138N mice were left untreated or treated with zVAD and/or Nec-1 for 1 hr prior to stimulation with LPS. LPS and zVAD treatment of WT BMDMs induced cell death that was prevented by Nec-1 pretreatment or RIPK3-deficiency (Figure 7). We found that Ripk1D138N/D138N BMDMs were also protected from LPS–induced necroptosis (Figure 7). RIPK1 kinase inactive or Nec-1 pre-treated WT BMDMs were similarly resistant to TLR3-induced necroptosis via poly(I:C)/zVAD stimulation (data not shown)40. Thus, RIPK1 kinase activity is essential for LPS- and poly(I:C)–induced necroptosis in primary BMDMs in vitro40.

Figure 7. RIPK1 kinase activity and RIPK3 mediate LPS/zVAD-induced necroptosis in primary macrophages in vitro. Primary BMDMs generated from WT, Ripk1D138N/D138N, and Ripk3−/− mice were treated with LPS (20 ng/ml), zVAD (20 μM), and Nec-1 (30 μM) for 20 hours and cell viability was measured using MTS assay (n = 3 biological replicates). Error bars represent SEM.
Discussion

Here we demonstrate that RIPK1 kinase activity is required for TNF/Smac/zVAD-induced necrosome formation and induction of necroptotic death downstream of TNFR1 or TLR3/4 receptors. Additionally, we show that TNF/Smac-but not TNF/Cx-induced apoptosis requires RIPK1 kinase activity. By generating RIPK1 kinase inactive mice and utilizing their MEFs and BMDMs, we were able to use endogenous RIPK1 kinase inactive protein instead of relying on in vitro reconstitution or small molecule inhibitors to implicate RIPK1 kinase activity in regulating necroptosis43,44.

RIPK1 and RIPK3 RHIM and kinase activities were both required for TNF/Cx/zVAD or TNF/Smac/zVAD-induced necrosome formation and necroptotic death in these studies, with a proposed role for auto- and trans-phosphorylation of RIPK1 and RIPK3 inducing conformational changes promoting oligomerization and amyloid formation43,44. Using RIPK1 kinase inactive MEFs, we demonstrate that RIPK1 kinase activity is required for efficient necrosome complex formation upon TNF/Smac/zVAD treatment (Figures 4 and 5). In addition to detecting a RIPK1/RIPK3/caspase-8 interaction with caspase-8 immunoprecipitation, we immunoprecipitated FADD, demonstrating a FADD/RIPK1/caspase-8 complex forms in a RIPK1 but not RIPK3 kinase-dependent manner after TNF/Smac/zVAD stimulation (data not shown). Due to interference from Ig light and heavy chain, we could not clearly detect FADD in the caspase-8 immunoprecipitated complex or RIPK3 in the FADD-associated complex. To mitigate this, follow up studies could
be done with light- or heavy-chain specific secondary antibodies. However, contrary to what was reported, we did not detect a constitutive FADD/RIPK3 interaction in WT MEFs (data not shown)\textsuperscript{43,181}.

In the absence of RIPK3, a stable TNF/Smac/zVAD-induced caspase-8/RIPK1 interaction required RIPK1 kinase activity, but these cells were resistant to necroptosis due to lack of RIPK3 expression (Figure 5A and data not shown). As reported by He et al.\textsuperscript{44}, when present RIPK3 was recruited into this complex II, but in a RIPK1 kinase-specific manner. RIPK3 kinase inhibitors seemed to potentiate rather than inhibit complex formation, but RIPK3 kinase activity was required for induction of necroptosis downstream of complex formation (Figures 5A and 6B). Similarly, RIPK3 kinase inhibitors did not block a RIPK1/RIPK3 interaction while RIPK1 kinase activity was required (Figure 4B). \textit{In vitro} kinase assays have demonstrated that RIPK3 can phosphorylate RIPK1, but RIPK1 phosphorylation of RIPK3 was not detected\textsuperscript{43,87}. However, necrosome formation is not affected by RIPK3 kinase inhibition, and RIPK1 may be phosphorylated upon TNF/Smac/zVAD-treatment in a RIPK3-independent manner. This is most likely auto-phosphorylation, as the slower migrating band is absent when cells are treated with RIPK1 kinase inhibitors (Figure 4B). Therefore, a conformational change in RIPK1 and activation of its kinase activity may initiate recruitment of RIPK3 and mediate their initial RHIM-dependent necroptosome interaction. While RIPK3 kinase activity appears dispensable for this interaction in MEFs, it is
required to activate RIPK3 for downstream recruitment and activation of MLKL to induce necroptotic death.
Methods

Immunoblotting

Mouse embryonic fibroblasts were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% NP40, 0.25% deoxycholate, 0.1% SDS, 1 mM EDTA), supplemented with protease inhibitors (Roche Applied Science), supplemented with sodium orthovanadate (1 mM), phenylmethylsulfonyl fluoride (PMSF, 1 mM), and dithiothreitol (DTT, 1 mM). For coimmunoprecipitation, cells were lysed in an endogenous lysis buffer (0.5M Tris-HCl pH 7.6, 0.5M NaCl, 0.1M EDTA, 1% Triton X-100, 0.5M NaF, and 0.5M sodium pyrophosphate) supplemented with complete protease inhibitor cocktail and phosphatase inhibitors (Roche). Total protein was run on a 10% SDS-PAGE gel, transferred to a PVDF membrane (Invitrogen) and probed with antibodies to caspase-8, RIPK1, RIPK3, cFLIP, and ERK1/2. Membranes were developed with Clarity Western ECL Substrate (BioRad).

Cell death assays

Wild-type, Ripk1<sup>D138N/D138N</sup>, Ripk1<sup>-/-</sup>, and Ripk3<sup>-/-</sup> MEFs were pre-treated with 10 μg/ml cycloheximide, 20 μM Zvad (Enzo), 10 μM Smac mimetic (ChemiTek), or 30 μM Nec-1 and then treated with 10 ng/ml mTNF (R&D Systems). Wild-type, Ripk1<sup>D138N/D138N</sup>, or Ripk3<sup>-/-</sup> BMDMs were treated with 20 μM zVAD or 30 μM Nec-1 and then with 20 ng/ml LPS. Metabolic activity was assayed after 20 hours by addition of MTS reagent (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega) and measured using a Beckman Coulter DTX880 plate reader. Absorbance values at 535nm were normalized to control.
CHAPTER III

RIPK1 kinase inactive mice are resistant to TNF- but not LPS-induced shock


*All experiments and data analysis in Figures 8 to 14 were performed by Matija Zelic, with contribution from Nicole Hermance (Figures 8, 10, and 11C and D) and Justine Roderick (Figures 8 and 9)
Introduction

As demonstrated in Chapter 2, RIPK1 kinase activity is required for TNF-induced necroseome formation and TNF- and TLR3/4-mediated necroptosis in vitro. RIPK1 and RIPK3 activation results in phosphorylation of MLKL, leading to membrane permeabilization and the release of DAMPs\(^{289,290}\). ATP, HMGB1, heat shock proteins, and other DAMPs are recognized by innate immune cells and can initiate or further amplify proinflammatory cytokine and chemokine production; thus, necroptosis is thought to be a more proinflammatory form of cell death compared to apoptosis\(^{67}\). RIPK1 has also been suggested to mediate cytokine/chemokine transcription or translation in certain settings\(^{24,103,277,278}\). Thus, we wanted to test the physiologic role of RIPK1 kinase activity in a mouse model where inflammation and necroptosis had been implicated.

An acute inflammatory model of sterile sepsis used to model lethal systemic inflammatory response syndrome (SIRS) is TNF- or TNF/zVAD-induced shock, which was reported to depend on necroptosis due to protection in RIPK3-deficient mice\(^{97}\). Additionally, caspase inhibition seems to sensitize mice in this shock model\(^{97,298}\). However, the role of RIPK1 kinase activity in this model is unclear as Nec-1 was found to both sensitize and protect mice from TNF-induced SIRS\(^{97-99}\). Another sepsis model involves LPS administration, thus mimicking Gram-negative bacterial bloodstream infection, and LPS is known to potently induce TNF production in vitro and in vivo\(^{299,300}\). Here we demonstrate that RIPK1 kinase
inactive mice are resistant to TNF- and TNF/zVAD-induced shock but littermates are not resistant to LPS-induced shock.
Results

*RIPK1 D138N mice are resistant to TNF- or TNF/zVAD-induced shock*

TNF administration to mice mimics the histopathological and pathophysiologic changes associated with septic shock. We challenged Ripk1<sup>D138N/D138N</sup> mice, as well as WT and Ripk3<sup>-/-</sup> mice, with 9 μg TNF, a dose determined previously in our hands to induce death in 100% of treated WT mice. In contrast to WT controls, Ripk1<sup>D138N/D138N</sup> and Ripk3<sup>-/-</sup> mice were protected from hypothermia, and all of the animals survived the TNF challenge (Figure 8A and B). RIPK1 kinase inactive and RIPK3-deficient mice were similarly protected from TNF/zVAD-induced shock, which increased the kinetics and penetrance of lethality compared to TNF (Figure 8C and D). In these studies, we used age-matched males, but the WT and RIPK1 kinase inactive mice were not littermates. To determine sensitivity in littermates and whether one kinase inactive RIPK1 allele was sufficient for protection, we treated WT, Ripk1<sup>D138N/+</sup> and Ripk1<sup>D138N/D138N</sup> mice with a 4-fold higher dose of TNF combined with zVAD. While about half of the RIPK1 kinase inactive mice succumbed to this high-dose TNF/zVAD-induced shock, they were significantly protected compared to WT mice (Figure 9). Interestingly, one kinase inactive allele of RIPK1 was sufficient to mediate protection from TNF/zVAD-induced shock, indicating that the relevant cell types in this model are sensitive to RIPK1 kinase inactive gene dosage or that the D138N mutation has dominant negative activity (Figure 9). Thus, expression of a kinase-inactive form of RIPK1 was sufficient to provide protection from TNF-induced...
hypothermia and mortality in the presence or absence of caspase inhibition and we confirmed the protective role of RIPK3 (Figures 8 and 9). Collectively, these genetic studies suggest that the mortality induced by TNF in this shock model reflects RIPK1- and RIPK3-mediated necroptotic death in vivo.

**Figure 8.** Ripk1<sup>D138N/D138N</sup> and RIPK3-deficient mice are protected from TNF- or TNF/zVAD-induced shock, a mouse model of systemic inflammatory response syndrome. Body temperatures and survival of age- and sex-matched wild-type, Ripk1<sup>D138N/D138N</sup>, and Ripk3<sup>−/−</sup> mice injected with mTNF (A and B) (p < 0.0001) or mTNF and zVAD (C and D) (p < 0.0001).
Figure 9. *Ripk1*<sup>D138N/+</sup> littermate mice are protected from high-dose TNF/zVAD-induced shock due to gene dosage sensitivity or dominant negative kinase activity. Body temperature (A) and survival (B) of wild-type, *Ripk1*<sup>D138N/+</sup>, and *Ripk1*<sup>D138N/D138N</sup> littermates injected with high dose (1.25mg/kg) mTNF and zVAD.
**RIPK1 kinase inactive littermates are susceptible to LPS-induced shock**

Since RIPK1 kinase inactive mice were resistant to TNF-induced shock and LPS administration induces TNF production *in vivo*, we injected age-matched male WT and RIPK1 kinase inactive mice with LPS. Although *Ripk1<sup>D138N/D138N</sup>* mice did undergo hypothermia in response to a 5 or 10mg/kg dose of LPS, they were significantly protected from LPS-induced hypothermia and lethality compared to WT controls (Figure 10). However, when we increased the dose of LPS to 20mg/kg, *Ripk1<sup>D138N/D138N</sup>* mice underwent hypothermia and succumbed to LPS-induced shock at similar rates as WT mice (Figure 11C and D). Thus, RIPK1 kinase inactive males may be partially resistant to lower doses of LPS.

A big problem in mouse models is the heterogeneity of the gut microbiome and passenger mutations which are present in various strains of mice, in some cases even if mice are back-crossed on to the same strain<sup>301,302</sup>. Littermate controls are therefore crucial to use, especially in mouse models of sepsis. To confirm the protection of RIPK1 kinase inactive mice at lower doses of LPS administration, we administered 10mg/kg of LPS to WT and *Ripk1<sup>D138N/D138N</sup>* littermates. However, no differences between WT and RIPK1 kinase inactive mice with regards to LPS-induced hypothermia were observed (Figure 11A and B). Therefore, unlike the critical role of RIPK1 kinase activity in mediating TNF-induced shock (Figures 8 and 9), RIPK1 kinase inactive mice do not seem to ameliorate LPS-induced morbidity (Figure 11), as was reported recently by a separate group<sup>227</sup>.
Figure 10. *Ripk1*<sup>D138N/D138N</sup> non-littermate mice are partially resistant to low dose LPS-induced shock. Body temperatures (A and C) and survival (B and D) of age- and sex-matched wild-type and *Ripk1*<sup>D138N/D138N</sup> mice injected with 5mg/kg LPS (A and B) or 10mg/kg LPS (C and D).
Figure 11. RIPK1 kinase inactive mice are susceptible to LPS-induced shock similar to their WT littermate controls. Body temperatures (A, C) and survival (B, D) of littermate (A and B) or age- and sex-matched (C and D) wild-type and Ripk1<sup>D138N/D138N</sup> mice injected with 10mg/kg LPS (A and B) or 20mg/kg LPS (C and D).
**RIPK1 kinase inactive mice exhibit impaired upregulation of LPS-induced cytokines and chemokines in vivo**

Since RIPK1 kinase inactive non-littermate mice were protected from lower dose (5mg/kg) LPS administration, we analyzed whether these mice had reduced levels of LPS-induced cytokines and chemokines *in vivo* compared to susceptible WT mice. Similar increases in IL-6, CXCL2, CCL2 and CCL5 levels were observed in WT and *Ripk1<sup>D138N/D138N</sup>* mice at both early (1.5hr) and later (7hr) time points (Figure 12). However, levels of TNF, CXCL1, CCL3, CCL4, IL-1β, IL-17α, IFN-γ, and GM-CSF were reduced in RIPK1 kinase inactive mice compared to LPS-treated WT mice at both the early and later time points. These data suggest that RIPK1 kinase activity may modulate LPS-induced production of certain cytokines and chemokines *in vivo*. Additionally, the resistance of non-littermate RIPK1 kinase inactive mice to lower doses of LPS may in part be mediated by reduced cytokine and chemokine levels *in vivo*.
Figure 12. *Ripk1<sup>D138N/D138N</sup>* mice are impaired in their ability to upregulate an LPS-driven proinflammatory cytokine and chemokine signature *in vivo*. Plasma cytokine and chemokine levels in WT and *Ripk1<sup>D138N/D138N</sup>* mice 1.5 and 7 hours after intraperitoneal LPS (5mg/kg) administration (n = 4-6) Error bars represent SEM.
**RIPK1 kinase activity partially impairs LPS/zVAD-induced cytokine and chemokine production in vitro**

To test whether RIPK1 kinase is required for transcriptional activation of inflammatory cytokines/chemokines, we isolated primary BMDMs from WT and *Ripk1*^{D138N/D138N} mice and stimulated them *in vitro* with LPS or LPS/zVAD for 6 hours. LPS- or LPS/zVAD-induced *Il6* and *Ccl5* levels were normally increased in *Ripk1*^{D138N/D138N} BMDMs, consistent with their upregulation *in vivo* in *Ripk1*^{D138N/D138N} mice (Figures 12 and 13). Interestingly, we found that the kinase activity of RIPK1 was required for the induction of *Tnf* and *Csf2* (GM-CSF), but not *Cxcl1* and *Il-1β* in response to LPS/zVAD treatment (Figure 13). However, *in vivo* the secretion of these 4 cytokines/chemokines was reduced in LPS-treated RIPK1 kinase inactive mice, suggesting that perhaps RIPK1 affects CXCL1 and IL-1β translation or processing, but not transcriptional upregulation. Meanwhile, LPS-induced responses were similar between WT and RIPK1 kinase inactive BMDMs for the cytokines/chemokines analyzed. Thus, RIPK1 kinase activity may contribute to LPS/zVAD-induced TNF and GM-CSF transcription in primary BMDMs *in vitro*, and may be responsible for the reduction in cytokine and chemokine levels during LPS-induced shock *in vivo*.

We did not detect significant differences in LPS- or LPS/zVAD-induced TNF or IL-6 secretion at 6 or 22 hours between WT and *Ripk1*^{D138N/D138N} BMDMs (Figure 14A). Even though TNF secretion was consistently decreased in LPS- or LPS/zVAD-treated RIPK1 kinase inactive BMDMs, the difference was not
significant (Figure 14A) while LPS/zVAD-induced Tnf upregulation was significantly reduced in RIPK1 kinase inactive macrophages (Figure 13). Similar to LPS-induced Il-1β expression, IL-1β secretion levels were similar in WT and RIPK1 kinase inactive BMDMs primed with LPS and stimulated with polydA:dT, silica, or ATP (Figure 14B). Thus, RIPK1 kinase activity is dispensable for canonical NLRP3 inflammasome activation.
Figure 13. RIPK1 kinase activity is required for LPS/zVAD-induced Tnf and Csf2 expression in primary macrophages in vitro. Quantitative RT-PCR analysis of TNFa, Il6, Cxcl1, Ccl5, Csf2, and Il1b expression in WT and Ripk1D138N/D138N primary macrophages treated with LPS or LPS/zVAD for 6 hours (n = 3 mice). Error bars represent SEM.
Figure 14. *Ripk*<sub>1</sub><sup>D138N/D138N</sup> primary macrophages do not contribute to LPS or LPS/zVAD-induced TNF, IL-6, or IL-1β up-regulation *in vitro*. (A) Secretion of TNF and IL-6 in WT and *Ripk*<sub>1</sub><sup>D138N/D138N</sup> primary macrophages 6 and 22 hours after addition of LPS or LPS/zVAD (n = 3 mice). (B) Secretion of IL-1β in WT and *Ripk*<sub>1</sub><sup>D138N/D138N</sup> primary macrophages 6 hours after addition of polydA:dT or silica, or 45 minutes after ATP addition. Macrophages were primed for 3 hours with LPS (n = 3 mice).
Discussion

Here we demonstrate that RIPK1 kinase activity is required for TNF and TNF/zVAD-induced shock in mice, but seems dispensable for LPS-induced shock, at least in littermates. Additionally, we show that non-littermate RIPK1 kinase inactive mice that are partially resistant to LPS-induced shock fail to upregulate some, but not all LPS-induced cytokines and chemokines examined. In BMDMs in vitro, however, we find RIPK1 kinase inactive cells impair only Tnf and Csf2 transcriptional upregulation in response to LPS/zVAD but not LPS treatment.

We demonstrate the requirement of both RIPK1 kinase activity and RIPK3 in a mouse model of TNF- or TNF/zVAD-induced shock. Caspase inhibition sensitized mice to TNF-induced shock, as reported previously, suggesting RIPK1 and RIPK3-driven necroptosis plays an integral part in inducing the shock and lethality. Though we did not use littermates in Figure 8, we used age-matched males that were co-housed for a few weeks prior to treatment when possible. Importantly, when we treated littermates with zVAD and a 4-fold higher dose of TNF, we still saw significant protection in RIPK1 kinase inactive mice compared to WT controls (Figure 9). Surprisingly, heterozygous RIPK1 kinase inactive mice (Ripk1<sup>D138N/+</sup>) were as resistant as their homozygous kinase inactive littermates, demonstrating that the D138N mutation may act as a dominant negative in cell types crucial in mediating TNF-induced signaling and lethality in this shock model.

The importance of littermate controls was exemplified by our LPS studies. We found that RIPK1 kinase inactive mice littermates were sensitized to LPS-
induced hypothermia similarly to WT mice when treated with 10mg/kg LPS, in agreement with a recent report\textsuperscript{227}. While this dose did not induce lethality, non-littermate RIPK1 kinase inactive were similarly sensitive as WT mice to lethality induced by 20mg/kg LPS administration (Figure 11). Although RIPK1 kinase inactive mice are resistant to TNF-induced shock and LPS administration induces TNF production, RIPK1 kinase inactive littermates are susceptible to LPS-induced shock. However, our initial experiments performed in non-littermate, age-matched males indicated that RIPK1 kinase inactive mice were partially resistant to LPS doses of 5mg/kg and 10mg/kg compared to WT mice, and these doses induced lethality in more than 50% of WT controls (Figure 10). Thus, we observed differences in LPS-induced shock and dose sensitization in both WT and RIPK1 kinase inactive mice once we repeated the experiment in littermates. Part of the dose-dependent sensitization difference could also be due to varying potency in different LPS batch preparations.

In non-littermates treated with 5mg/kg LPS we observed a reduction in various LPS-induced cytokines and chemokines in RIPK1 kinase inactive mice (Figure 12), consistent with a recent study showing RIPK1 kinase activity promotes LPS-induced inflammatory responses\textsuperscript{277}. Consistent reductions in LPS-treated \textit{Ripk1}\textsuperscript{D138N/D138N} mice were observed for TNF, GM-CSF, and IL-1\textbeta levels (Figure 12), indicating that RIPK1 kinase activity may regulate their transcription or translation. Indeed, the relative expression of \textit{Tnf} and \textit{Csf2} was reduced in LPS/zVAD- but not LPS-treated RIPK1 kinase inactive BMDMs (Figure 13), while
TNF secretion was not significantly reduced in LPS- or LPS/zVAD-treated BMDMs (Figure 14A). The discrepancy between LPS/zVAD-induced TNF transcriptional and translational responses \textit{in vitro} is unclear but may reflect differences in kinetics and/or a partial requirement for RIPK1 kinase activity. Meanwhile, the induction of \textit{Il-1β} expression was unaffected by lack of RIPK1 kinase activity (Figure 13) and canonical NLRP3 inflammasome activation and IL-1β secretion was not impaired in RIPK1 kinase inactive BMDMs (Figure 14B), although LPS-induced IL-1β levels \textit{in vivo} were reduced in \textit{Ripk1}^{D138N/D138N} mice (Figure 12). Perhaps RIPK1 regulation in other cell types contributes to differences observed in mice. Alternatively, the canonical NLRP3 inflammasome activators we used \textit{in vitro} may not reflect the mechanism of IL-1β processing and secretion in response to lethal LPS-induced shock.
Methods

Mice

*Ripk1*<sup>D138N/D138N</sup> knock-in mice were generated by mutating the conserved aspartate (D) at position 138 to asparagine (D138N). The *Ripk1*<sup>D138N</sup> construct was introduced into Bruce 4 embryonic stem cells derived from C57BL/6 mice. Mice were maintained at the specific pathogen–free animal facilities of the University of Massachusetts Medical School and the Institute for Genetics at the University of Cologne. All animal procedures were conducted in accordance with national and institutional guidelines. Sex- and age-matched mice were used in all experiments.

TNF- and LPS-induced shock experiments

Age- and sex-matched wild-type, *Ripk1*<sup>D138N/D138N</sup> and *Ripk3*<sup>−/−</sup> mice were injected intravenously with 9 μg mTNF only or with zVAD-fmk (16.7 mg/kg). LPS was injected intraperitoneally at varying doses (5, 10, or 20mg/kg). Body temperature and survival were monitored.

Cytokine Analysis

Plasma cytokines were measured using a 12-plex protein/peptide multiplex analysis (Luminex Technology) conducted by the National Mouse Metabolic Phenotyping Center at the University of Massachusetts Medical School. Cytokines and chemokines that were below the limit of detection were assigned a value of zero. Blood was collected via cardiac puncture in a heparinized needle and spun down to isolate plasma. The samples were stored at -80°C until analysis. IL-6
(eBioscience), TNF, and IL-1β secretion by cultured primary cells was determined by ELISA.

**Gene expression Analysis**

Primary BMDMs were treated with LPS (10ng/mL) and 50 μM zVAD (Enzo) for 6 hours. Total cellular RNA was isolated using an RNeasy Mini Kit (QIAGEN). cDNA was prepared using the Superscript First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and run on the AB7300 Detection System (Applied Biosystems). Relative gene expression was normalized to Actin and determined using the ΔΔCT method, with the following primer sequences:

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Direction</th>
<th>Primer sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>For</td>
<td>CGAGGCCCGAGAGGCAAGGAG</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>CGTTGGCCCTAGGGTGCAAG</td>
</tr>
<tr>
<td>Tnf</td>
<td>For</td>
<td>CAGTTCTATGGCCAGACCT</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>CGGACTCCGAAAGTCTAAG</td>
</tr>
<tr>
<td>Il6</td>
<td>For</td>
<td>AACGATGATGCACCTGAGA</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>GAGCATGGAAATGGGTA</td>
</tr>
<tr>
<td>Ccl5</td>
<td>For</td>
<td>GCCCACGTCAGAAGATTTTCTA</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>ACACACTTGCGGTTCTTCTC</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>For</td>
<td>CTGGGATTCACCTCAAGAATCAT</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>CAGGGTCAAGGCAAGCCTC</td>
</tr>
<tr>
<td>Csf2 (GM-CSF)</td>
<td>For</td>
<td>GGCTTGGAAAGCATGTAGAGG</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>GGAGAAGCTCGTAGAGAAGACTT</td>
</tr>
<tr>
<td>Il-1β</td>
<td>For</td>
<td>GCCCATCCTCAGTGACTCAT</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>AGGCCACAGGATTTTGTCG</td>
</tr>
</tbody>
</table>
CHAPTER IV

RIP kinase 1-dependent endothelial necroptosis underlies lethal TNF/zVAD-induced systemic inflammatory response syndrome

*Data in this chapter are part of a manuscript in revision at JCI: Zelic M, Roderick JE, O'Donnell JA, Lehman J, Lim SE, Janardhan HP, Trivedi CM, Pasparakis M, and Kelliher MA. RIP kinase 1-dependent endothelial necroptosis underlies systemic inflammatory response syndrome. J. Clin. Inv.

*All experiments and data analysis shown in Figures 15 to 28 were performed by Matija Zelic, with contribution from Jo O'Donnell (Figures 17, 19, and 20), Justine Roderick (Figures 17, 18, 20, 22, and 27), Jesse Lehman (Figures 17 and 20), Harish Janardhan (Figure 24), and Nicole Hermance (Figure 23)
Introduction

The recent generation of kinase inactive Ripk1\textsuperscript{D138N/D138N} and Ripk1\textsuperscript{K45A/K45A} mice has elucidated the role of RIPK1 kinase activity in necroptosis and in mouse models of acute and chronic inflammatory disease\textsuperscript{24,40,41}. As demonstrated in Chapter 3, and similar to Ripk3\textsuperscript{-/-} mice, RIPK1 kinase inactive Ripk1\textsuperscript{D138N/D138N} mice are protected from TNF- or TNF/zVAD-induced acute shock, a model of SIRS\textsuperscript{40,97} (Figures 8 and 9). SIRS can be triggered by sterile inflammation including injury from burns, trauma, or acute pancreatitis, and upon infection can lead to sepsis and septic shock\textsuperscript{303,304}. It has long been hypothesized that the “cytokine storm” drives shock, with excess production of proinflammatory cytokines such as TNF\textgreek{a} and IL-1\textbeta triggering vascular dysfunction and organ injury\textsuperscript{303}. However, attempts over the last couple of decades to block TNF or IL-1R signaling in septic patients have largely failed to provide a survival benefit\textsuperscript{305–307}.

Although necroptosis has recently been implicated in the pathogenesis of TNF-induced shock and SIRS\textsuperscript{24,40,41,97}, the precise cell types undergoing necroptosis and the relative contribution of the “cytokine storm” remain unclear. Here, we demonstrate that proinflammatory cytokine/chemokine levels do not correlate with hypothermia or survival in this mouse SIRS model, and that non-hematopoietic RIPK1 kinase inactive cells mediate resistance to TNF- or TNF/zVAD-induced shock. Additionally, we show that WT liver endothelial cells undergo MLKL activation and cell death, indicating that endothelial cell necroptosis
contributes to increased vascular permeability, coagulation, and organ injury observed in TNF/zVAD-treated WT but not RIPK1 kinase inactive mice.
Results

**RIPK1 kinase activity regulates cytokine/chemokine production and neutrophil infiltration in response to TNF and TNF/zVAD**

TNF shock mimics systemic SIRS, which is thought to be driven by the proinflammatory “cytokine storm”. To assess the role of RIPK1 kinase activity in the “cytokine storm”, we analyzed proinflammatory cytokine and chemokine levels in the plasma of TNF- or TNF/zVAD-treated mice. Similar increases in CXCL1, CCL3, CCL4, CCL5, and IL-6 levels were observed in Ripk1<sup>D138N/D138N</sup> and WT mice at early time points after TNF administration (Figure 15A). However, CXCL2 levels were consistently decreased in Ripk1<sup>D138N/D138N</sup> mice, suggesting that RIPK1 kinase activity may be required for optimal TNF-induced CXCL2 production in vivo (Figure 15A). Cytokine and chemokine levels were consistently reduced at 7 hours in RIPK1 kinase inactive mice compared to TNF-treated WT mice (Figure 15A). The addition of the pan-caspase inhibitor zVAD sensitizes WT mice to TNF shock and decreases time to lethality<sup>40,97,298</sup>. To ascertain whether TNF/zVAD sensitization correlates with increased proinflammatory cytokine levels, we compared TNF- and TNF/zVAD-induced plasma cytokine/chemokine levels at 2 hours. Interestingly, WT mice treated with TNF/zVAD exhibited significantly elevated plasma cytokine and chemokine levels compared to treatment with TNF alone, especially for CXCL2, CCL3, CCL4, and IL-6 (Figure 15B). IL-1β and IL-17α levels, which are found increased in septic patients<sup>308,309</sup>, were elevated systemically in WT mice treated with TNF/zVAD but not TNF (Figure 15B). While
the addition of zVAD amplified cytokine and chemokine levels in WT mice, \( \textit{Ripk1}^{D138N/D138N} \) mice exhibited significantly lower plasma levels of proinflammatory cytokines and chemokines (Figure 15B). Overall, these results suggest that the plasma proinflammatory cytokine/chemokine levels correlate with the kinetics of hypothermia and lethal shock. Furthermore, these data suggest that TNF/zVAD sensitizes mice to necroptotic death \textit{in vivo}, which is postulated to be proinflammatory due to DAMP release\(^{67}\).

Recently, the kinase activity of RIPK1 has been implicated in the transcriptional regulation of certain cytokines and chemokines in response to TNF/zVAD or LPS/zVAD treatment\(^{24,103,277,278}\). To test whether RIPK1 kinase is required for transcriptional activation of inflammatory cytokines/chemokines, we isolated primary BMDMs from WT and \( \textit{Ripk1}^{D138N/D138N} \) mice and stimulated them \textit{in vitro} with TNF or TNF/zVAD. We found that the kinase activity of RIPK1 was required for the induction and production of CXCL1, CXCL2, and IL-6, but not CCL5, in response to TNF/zVAD (Figure 16, A and B). Interestingly, \( \textit{Ripk1}^{D138N/D138N} \) BMDMs also exhibited reduced CXCL2 expression and secretion when treated with TNF alone, indicating that RIPK1 kinase activity is necessary for optimal TNF-induced \( Cxcl2 \) upregulation \textit{in vitro} (Figure 16, A and B). Thus, RIPK1 kinase activity contributes to TNF and TNF/zVAD-induced cytokine and chemokine transcription in primary BMDMs \textit{in vitro}, and may be responsible for reduced cytokine and chemokine levels during TNF shock \textit{in vivo}. 
Figure 15. *Ripk1*<sup>D138N/D138N</sup> mice exhibit impaired cytokine and chemokine production in response to TNF and TNF/zVAD administration *in vivo*. (A) Plasma cytokine and chemokine levels in WT and *Ripk1*<sup>D138N/D138N</sup> mice 2 and 7 hours after intravenous TNF administration (n = 8-9) or (B) 2 hours after intravenous TNF or TNF/zVAD administration (n = 6-9). TNF-induced levels are repeated from (A). Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, by 2-tailed Student’s *t* test.
Figure 16. RIPK1 kinase activity contributes to TNF- and TNF/zVAD-induced CXCL1 and CXCL2 chemokine and IL-6 cytokine production in vitro. (A) Quantitative RT-PCR analysis of Cxcl1, Cxcl2, Il6, and Ccl5 expression in WT and Ripk1^{D138N/D138N} primary macrophages treated with TNF or TNF/zVAD for 2 or 6 hours (n = 3 mice). Graphs show relative mRNA expression normalized to Actin. (B) Secretion of CXCL1, CXCL2, IL-6 and CCL5 as determined by ELISA in WT and Ripk1^{D138N/D138N} primary macrophages 6 or 22 hours after addition of TNF or TNF/zVAD (n = 3 mice).
To determine whether reductions in cytokine/chemokine levels in RIPK1 kinase inactive mice result in decreased inflammatory cell mobilization and infiltration, we analyzed peripheral blood, bone marrow, spleen, lung, and liver for evidence of inflammatory macrophage and neutrophil recruitment in response to TNF or TNF/zVAD administration. Systemic TNF administration mobilized inflammatory macrophages and neutrophils from the spleens and bone marrow of WT and Ripk1<sup>D138N/D138N</sup> mice (Figure 17, A and B). However, more neutrophils were detected in Ripk1<sup>D138N/D138N</sup> spleen and bone marrow compared to TNF-treated WT mice (Figure 17, A and B). TNF-induced inflammatory monocyte recruitment to the blood and similar numbers of inflammatory monocytes/macrophages were detected in the lungs and liver of TNF-treated WT and Ripk1<sup>D138N/D138N</sup> mice (Figure 17, C-E). Consistent with reduced levels of neutrophil chemoattractant CXCL2 (Figure 15A), TNF-treated Ripk1<sup>D138N/D138N</sup> mice exhibited decreased neutrophil mobilization from the bone marrow and spleen into the blood. However, inflammatory cell infiltration in lung and liver was similar for WT and Ripk1<sup>D138N/D138N</sup> mice treated with TNF (Figure 17, C-E). Although TNF/zVAD administration to WT mice results in a more robust cytokine/chemokine response (Figure 15B), this did not result in significant increases in inflammatory cell mobilization into blood, lung or liver compared to treatment with TNF only (Figure 17, C-E). Although TNF/zVAD treatment significantly increased neutrophil numbers in the spleen and bone marrow,
*Ripk1<sup>D138N/D138N</sup>* mice exhibited reduced neutrophil infiltration into lung and liver (Figure 17). Activated neutrophils can release proteases and ROS, thereby damaging organs and further increasing cytokine production<sup>310</sup>. Thus, the reduced cytokine/chemokine levels and neutrophil infiltration observed in TNF and TNF/zVAD-treated *Ripk1<sup>D138N/D138N</sup>* mice may be responsible for the protection.

Figure 17. Impaired recruitment of *Ripk1<sup>D138N/D138N</sup>* neutrophils to the lung and liver in response to TNF or TNF/zVAD administration. (A, B) Splenocytes and bone marrow cells were stained with antibodies, analyzed by flow cytometry and the number of inflammatory monocytes/macrophages or neutrophils determined. The number of cells for WT and *Ripk1<sup>D138N/D138N</sup>* mice left untreated (Unt.) or treated with TNF (7h) or TNF/zVAD (4h) (n = 3-5) is shown. (C) The number of inflammatory monocytes or neutrophils in peripheral blood of WT and *Ripk1<sup>D138N/D138N</sup>* mice is shown (n = 3-5). (D, E) The number of inflammatory macrophages or neutrophils in lung and liver of WT and *Ripk1<sup>D138N/D138N</sup>* mice left untreated (Unt.) or treated with TNF (7h) or TNF/zVAD (4h) (n = 4-5) is shown. *p<.05, **p<.01, ***p<0.001, ****p<0.0001, by 2-tailed Student’s t test.
**RIPK1 kinase activity in a non-hematopoietic lineage is responsible for TNF- and TNF/zVAD-induced lethality**

These data suggest that inflammatory cells mediate organ damage and predict that RIPK1 kinase inactive hematopoietic cells provide protection in this SIRS model. To test this, we generated reciprocal bone marrow chimeras by transplanting WT or Ripk1<sup>D138N/D138N</sup> bone marrow cells into lethally irradiated syngeneic recipients (Figure 18A). Two months post-reconstitution, RIPK1 kinase inactive donor-derived cells repopulated irradiated recipients as effectively as WT cells (Figure 19, A-C), indicating that RIPK1 kinase activity does not contribute to the cell-intrinsic survival function of RIPK1 in hematopoietic cells<sup>180,311</sup>. We next tested the TNF response and as expected, WT controls developed hypothermia in response to TNF (Figure 18B) or TNF/zVAD (Figure 18D) administration and succumbed to shock. Unexpectedly, Ripk1<sup>D138N/D138N</sup>→WT mice succumbed with similar kinetics as WT→WT controls in response to TNF (Figure 18C) or TNF/zVAD (Figure 18E) administration. Interestingly, WT→Ripk1<sup>D138N/D138N</sup> mice were completely protected from TNF- or TNF/zVAD-induced shock (Figure 18, B-E). These data reveal that a non-hematopoietic Ripk1<sup>D138N/D138N</sup> lineage(s) mediates protection from TNF- and TNF/zVAD-induced shock.
Figure 18. Non-hematopoietic RIPK1 kinase inactive cells mediate protection against lethal TNF- and TNF/zVAD-induced shock. (A) Experimental design. Body temperature (B, D) and Kaplan-Meier survival curves (C, E) after TNF- (B, C) or TNF/zVAD-induced shock (D, E) are depicted for lethally irradiated WT and Ripk1<sup>D138N/D138N</sup> mice reconstituted with WT or Ripk1<sup>D138N/D138N</sup> bone marrow cells. Error bars represent mean ± SEM. *p<.05, ***p<0.001, ****p<0.0001, by Mantel-Cox test.
Figure 19. Kinase inactive RIPK1 bone marrow cells effectively repopulate lethally irradiated hosts. Graphs depicting the percentage of donor-derived myeloid and lymphoid lineages in the spleen (A), bone marrow (B), and blood (C) at 8 weeks post-transplantation (n = 4-8 mice). Error bars represent mean ± SEM. IMs: Inflammatory Macrophages, DCs: Dendritic Cells.

**Cytokine levels and neutrophil infiltration do not correlate with survival**

To further examine any contribution of the “cytokine storm”, we measured proinflammatory cytokine and chemokine levels in the plasma of TNF-treated reconstituted mice. Comparable to what we observed in non-reconstituted mice, TNF treatment similarly upregulated cytokine and chemokine levels at 2 hours in all the bone marrow chimeras, notably revealing that RIPK1 kinase activity does not appear to regulate cytokine transcription during TNF shock (Figure 20A). While cytokine/chemokine levels were further elevated in WT controls 7 hours after TNF administration, unexpectedly both sensitive Ripk1D138N/D138N → WT and resistant WT → Ripk1D138N/D138N chimeras exhibited significant decreases in cytokine/chemokine levels (Figure 20A), demonstrating that cytokine/chemokine levels do not predict survival. These data also suggest that RIPK1 kinase inactive hosts lack a secondary trigger, potentially provided by the necroptosis of
nonhematopoietic cells, which may amplify proinflammatory signaling by feeding back on necroptosis-competent WT hematopoietic cells. Additionally, the data suggest that in sensitive $\text{Ripk}1^{D138N/D138N} \rightarrow \text{WT}$ mice, DAMPs released from necrototic WT non-hematopoietic cells fail to amplify cytokine/chemokine levels to levels observed in WT $\rightarrow$ WT mice, potentially because $\text{Ripk}1^{D138N/D138N}$ hematopoietic cells do not undergo necroptosis (Figure 20A). Furthermore, we found that TNF/zVAD treatment resulted in greater increases in cytokine/chemokine levels at 2 hours in both sensitive $\text{Ripk}1^{D138N/D138N} \rightarrow \text{WT}$ and resistant WT $\rightarrow$ $\text{Ripk}1^{D138N/D138N}$ chimeras compared to treatment with TNF only (Figure 21), suggesting that necroptosis amplifies proinflammatory cytokine/chemokine production. Additionally, maximal cytokine/chemokine production requires RIPK1 kinase activity in both hematopoietic and non-hematopoietic lineages, whereas a lack of RIPK1 kinase activity in a non-hematopoietic lineage provides protection.

We observed similar mobilization and infiltration of inflammatory monocytes/macrophages and neutrophils into the blood, lung and liver of all TNF-treated reconstituted groups (Figure 20, B-D). In fact, more inflammatory macrophages and neutrophils infiltrated the lung and liver, respectively, of protected WT $\rightarrow$ $\text{Ripk}1^{D138N/D138N}$ chimeras compared to sensitive $\text{Ripk}1^{D138N/D138N} \rightarrow$ WT mice. This finding suggests that a lack of RIPK1 kinase activity in either hematopoietic or non-hematopoietic cells does not abrogate TNF-induced inflammatory cell recruitment.
Figure 20. Proinflammatory cytokines and inflammatory cell recruitment do not correlate with survival in a TNF-induced model of shock. (A) Plasma cytokine and chemokine levels in reconstituted mice 2 and 7 hours after intravenous TNF administration (n = 4-7). Graphs depicting the number of inflammatory monocytes/macrophages and neutrophils in the blood (B), lungs (C), or liver (D) of lethally irradiated WT and Ripk1<sup>D138N/D138N</sup> mice reconstituted with WT or Ripk1<sup>D138N/D138N</sup> bone marrow cells as measured by flow cytometry 8 hours after systemic TNF administration (n = 4-5). Error bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, by two-way (A) or one-way ANOVA with post hoc Tukey test (B-D).
Figure 21. TNF/zVAD-treated RIPK1 kinase inactive hosts exhibit reduced cytokine and chemokine production compared to WT hosts reconstituted with RIPK1 kinase inactive hematopoietic cells. Plasma cytokine and chemokine levels in reconstituted mice 2 hours after intravenous TNF or TNF/zVAD administration (n = 3-7). TNF-induced levels are repeated from Figure 4A. Error bars represent mean ± SEM. *p<0.05, **p<0.01, by 2-tailed Student’s t test.

**TNF-induced increases in intestinal permeability require RIPK1 kinase activity**

Since RIPK1 kinase inactive hematopoietic cells proved not to be the critical lineage mediating protection from TNF shock (Figure 18), we examined other lineages affected by TNF administration. TNFR1 expression levels in intestinal epithelial cells (IECs) have been shown to correlate with TNF-induced increases in intestinal permeability and overall lethality in mice. To assess intestinal permeability, we measured the plasma fluorescence levels of orally gavaged FITC-conjugated dextran beads following TNF administration. WT mice exhibited a
significant increase in intestinal permeability but no increase in intestinal permeability was observed in Ripk1$^{D138N/D138N}$ mice upon TNF administration (Figure 22A). Although Ripk1$^{D138N/D138N}$ mice were protected from TNF-induced lethality, cleaved caspase-3 staining revealed similar numbers of apoptotic colonocytes in WT and RIPK1 kinase inactive mice (Figure 23B). Interestingly, WT mice had slightly elevated numbers of cleaved caspase-3 positive cells in the ileum, suggesting that ileal IECs may undergo RIPK1 kinase-mediated apoptosis in response to TNF (Figure 23A). Importantly, TNF/zVAD administration similarly suppressed ileal and colonic IEC apoptosis in WT and Ripk1$^{D138N/D138N}$ mice, suggesting that intestinal apoptosis is not responsible for the lethality observed in WT mice (Figure 23, A and B). Since intestinal leakage could disseminate the gut microflora systemically, activate TLR signaling, and amplify inflammatory cytokine production, we quantified bacterial growth in spleen and liver lysates from untreated and TNF-treated WT and Ripk1$^{D138N/D138N}$ mice (Figure 22B). Although TNF augmented intestinal permeability, we could not detect an increase in bacterial colonies from spleen and liver lysates when WT mice were treated with TNF (Figure 22B), indicating that changes in intestinal permeability were not sufficient to induce systemic bacteremia in this intravenous TNF shock model.
Figure 22. Non-hematopoietic RIPK1 kinase inactive cells mediate protection against TNF-induced intestinal and vascular hyperpermeability and coagulation. (A) FITC-labeled dextran beads were administered to WT and Ripk1^{D138N/D138N} mice by oral gavage 2 hours after TNF injection. Blood was isolated by cardiac puncture and the levels of FITC-dextran in plasma measured by fluorometry (n = 4-7). (B) Bacterial growth in spleen and liver lysates isolated from untreated WT and Ripk1^{D138N/D138N} mice or 10 hours after injection with 15 μg TNF (n = 3-6). (C) WT and Ripk1^{D138N/D138N} mice were injected with TNF and Evans Blue dye, organs were collected and the amount of dye extracted from the lung and liver was measured by spectrophotometry (n = 7-8). (D) Tissue factor levels were determined by ELISA in the plasma of TNF- or TNF/zVAD-treated WT and Ripk1^{D138N/D138N} mice (n = 3-7). (E) WT and Ripk1^{D138N/D138N} reconstituted mice were injected with TNF and Evans Blue dye, and the amount of dye extracted from the lung and liver was measured by spectrophotometry (n = 6-7). (F) Tissue factor levels in the plasma of reconstituted WT and Ripk1^{D138N/D138N} mice treated with TNF (n = 3-5). Error bars represent mean ± SEM. *p<0.05, **p<0.01, by 2-tailed Student’s t test (A-D) or one-way ANOVA with post hoc Tukey test (E-F).
Figure 23. Kinase inactive RIPK1 mice are modestly protected from TNF-induced ileal but not colonic intestinal epithelial apoptosis. Quantification of cleaved caspase-3 positive cells per 40x field in the ileum (A) and colon (B) of WT and Ripk1\textsuperscript{D138N/D138N} mice 2 hours after TNF or TNF-zVAD administration (n = 3). Error bars represent mean ± SEM. *p<0.05, **p<0.01, by 2-tailed Student’s t test.

**RIPK1 kinase activity is required for TNF-induced increases in vascular permeability and clotting**

In addition to increasing intestinal epithelial permeability, TNF stimulates vascular permeability and endothelial activation by upregulating the expression of adhesion molecules and by stimulating cytokine and chemokine production\textsuperscript{314,315}. To quantitatively assess TNF-induced vascular permeability changes we measured the extravasation of Evans Blue dye. TNF administration induced significant increases in vascular permeability, resulting in Evans Blue dye leakage into the lung and liver of WT mice, while Ripk1\textsuperscript{D138N/D138N} mice exhibited no detectable increase in vascular permeability (Figure 22C). Additionally, WT mice exhibited activation of the extrinsic coagulation cascade as evidenced by significant increases in plasma tissue factor levels following TNF or TNF/zVAD
administration (Figure 22D). To determine whether RIPK1 kinase activity in endothelial cells was responsible for the increased vascular permeability, we examined these vascular parameters in chimeric mice. WT → WT and Ripk1D138N/D138N → WT chimeras, which remain sensitive to TNF-induced shock, displayed increased vascular permeability in their lungs and liver (Figure 22E) and exhibited elevated tissue factor levels during TNF-induced shock (Figure 22F). Notably, WT → Ripk1D138N/D138N mice, which were protected from TNF-induced shock, showed no significant increase in TNF-induced lung or liver permeability and maintained basal levels of plasma tissue factor in response to TNF (Figure 22, E and F). These data suggest that RIPK1 kinase activity in endothelial cells is responsible for TNF-induced increases in vascular permeability and coagulation.

Consistent with these data, histological examination of the livers in TNF- and TNF/zVAD-treated WT mice revealed significant increases in blood vessel clotting compared to untreated controls (Figure 24, A and B). We observed neutrophil and erythrocyte adhesion to the endothelium and leakage of erythrocytes and leukocytes into the extravascular space due to increased vascular permeability (Figure 24A). Although we observed some evidence of neutrophil and erythrocyte adhesion and clotting in the TNF- and TNF/zVAD-treated Ripk1D138N/D138N liver sections, there was a significant reduction in clotting in the liver blood vessels (Figure 24, A and B). To quantify clotting function, we performed a prothrombin time test. Blood isolated from TNF-treated WT mice took significantly longer to clot compared to RIPK1 kinase inactive mice (Figure 24C), suggesting that coagulation
factors were consumed in TNF-treated WT mice due to activation of the extrinsic clotting cascade. These results demonstrate that lack of RIPK1 kinase activity in endothelial cells protects from TNF- and TNF/zVAD-induced increases in vascular permeability and coagulation.
Figure 24. Kinase inactive RIPK1 mice are protected from TNF- or TNF/zVAD-induced breaks in the liver endothelium and coagulation, and liver endothelial cell necroptosis induced by TNF/zVAD treatment. (A) Representative images of Hematoxylin and Eosin stained liver sections from WT or Ripk1^{D138N/D138N} mice left untreated or injected intravenously with TNF or TNF/zVAD (n = 3-4 mice). Scale bar: 100 μm (10x), 50 μm (40x). (B) Quantification of clotting observed in liver blood vessels of mice shown in (A). (C) Citrated plasma was isolated from WT and Ripk1^{D138N/D138N} mice 7 hours after TNF administration and clot formation time was measured by the prothrombin time test (n = 4 mice). (D) Representative images of endomucin stained liver sections from untreated, or TNF- or TNF/zVAD-treated WT and Ripk1^{D138N/D138N} mice (n = 3-4 mice). Scale bar: 50 μm (40x). (E) WT and Ripk1^{D138N/D138N} liver endothelial cells were isolated from untreated or TNF/zVAD-treated mice. A representative western blot showing protein lysates probed with antibodies to phospho-MLKL, MLKL and ERK1/2 is depicted (Lysates were generated and analyzed from 14 WT and 8 Ripk1^{D138N/D138N} TNF/zVAD-treated mice). Error bars represent mean ± SEM. **p<0.01, by 2-tailed Student’s t test.
**TNF-induced adhesion molecule expression does not require RIPK1 kinase activity**

We hypothesized that RIPK1 kinase activity may regulate vascular permeability by directly mediating endothelial cell activation. To test this possibility, we isolated primary endothelial cells from the lungs of WT and Ripk1<sup>D138N/D138N</sup> mice and measured ICAM-1, VCAM-1, E-selectin and P-selectin mRNA levels via real-time quantitative PCR. We detected no significant differences in TNF-induced ICAM-1, VCAM-1, E-selectin and P-selectin upregulation in WT and Ripk1<sup>D138N/D138N</sup> primary lung endothelial cells, indicating that RIPK1 kinase activity is not required for TNF-induced endothelial activation *in vitro* (Figure 25A). Endothelial cells upregulate neutrophil chemokines CXCL1 and CXCL2 in response to TNF stimulation<sup>315</sup>. Thus, we examined CXCL1 and CXCL2 expression and secretion but failed to detect any significant differences in endothelial chemokine production between WT and RIPK1 kinase inactive endothelial cells (Figure 25, A and B). We then assessed whether RIPK1 kinase activity influenced endothelial cell activation *in vivo*. After TNF or TNF/zVAD administration, we isolated RNA from WT and Ripk1<sup>D138N/D138N</sup> lung or liver and examined adhesion molecule and chemokine expression levels. We detected significant reductions in E- and P-selectin and in Cxcl2 expression in TNF or TNF/zVAD treated Ripk1<sup>D138N/D138N</sup> mice; however, no consistent differences were observed in ICAM-1 or VCAM-1 expression (Figure 26, A and B). Although chemokine differences were less pronounced in TNF-treated livers, TNF/zVAD
treatment resulted in significant increases in Cxcl2 expression in WT compared to Ripk1$^{D138N/D138N}$ liver (Figure 26B), perhaps reflective of increased plasma IL-1β and IL-17α levels$^{316-318}$. While decreased chemokine mRNA levels in Ripk1$^{D138N/D138N}$ lung and liver correlate with reduced neutrophil infiltration during TNF- and TNF/zVAD-induced shock (Figure 17, D and E), TNF- or TNF/zVAD-induced adhesion molecule upregulation in vivo does not appear to require RIPK1 kinase activity.
Figure 25. RIPK1 kinase activity is required for lung endothelial necroptosis induced by TNF/Smac/zVAD treatment but not for upregulation of adhesion molecules in endothelial cells in vitro. (A) Relative expression of ICAM-1, VCAM-1, E-selectin, P-selectin, Cxcl1 and Cxcl2 in WT and Ripk1^{D138N/D138N} primary lung endothelial cells treated with TNF for 1 or 4 hours (n = 3-4 mice). Graphs show relative mRNA expression normalized to Actin. (B) Secretion of CXCL1 and CXCL2 in WT and Ripk1^{D138N/D138N} primary lung endothelial cells 6 or 22 hours after addition of TNF (n = 3-4 mice). (C) Primary lung endothelial cells isolated from WT and Ripk1^{D138N/D138N} mice were treated with TNF (10 ng/ml), zVAD (20 μM), and/or Smac mimetic (1 μM) and cell viability was measured using MTS assay (n = 3 mice). Error bars represent mean ± SEM. *p<0.05, by 2-tailed Student’s t test.
Figure 26. Kinase inactive RIPK1 mice upregulate adhesion molecules and chemokines but exhibit decreased P-selectin and Cxcl2 expression in lung and liver treated with TNF or TNF/zVAD. Relative expression of E-selectin, P-selectin, VCAM-1, ICAM-1, Cxcl1 and Cxcl2 in WT and Ripk1D138N/D138N lungs (A) or livers (B) after TNF (4 or 8h) or TNF/zVAD (4h) treatment (n = 3-4 mice). Graphs show relative mRNA expression normalized to Actin. *p<0.05, **p<0.01, ***p<0.001, by 2-tailed Student’s t test.
**RIPK1 kinase activity mediates TNF-induced liver endothelial cell necroptosis**

Since the kinase activity of RIPK1 was not required for endothelial cell activation, we hypothesized that RIPK1 kinase-regulated endothelial cell death may be responsible for the increase in vascular permeability, coagulation and organ damage observed in WT mice. To test whether endothelial cells undergo necroptotic death, we isolated primary lung endothelial cells from WT and *Ripk1<sup>D138N/D138N</sup>* mice and treated them with concentrations of TNF, Smac mimetic and zVAD known to induce necroptosis in murine embryonic fibroblasts<sup>44</sup>. Primary lung endothelial cells isolated from WT mice did not undergo TNF/Smac-induced apoptosis or TNF/zVAD-induced necroptosis but were susceptible to necroptosis induced by TNF/Smac/zVAD treatment (Figure 25C). In contrast, *Ripk1<sup>D138N/D138N</sup>* endothelial cells were resistant to TNF/Smac/zVAD-induced necroptosis (Figure 25C). Collectively, these data demonstrate that RIPK1 kinase activity is required for TNF-induced endothelial necroptosis *in vitro* (Figure 25C), and for increased vascular permeability *in vivo* (Figure 22), implicating RIPK1 kinase-dependent endothelial cell necroptosis in SIRS.

To examine this possibility, we stained liver sections with endomucin, an endothelial cell marker<sup>319</sup>, after TNF or TNF/zVAD administration (Figure 24D). In treated WT mice we detected breaks in the endothelial barrier, indicating endothelial cell loss and/or detachment, and increased permeability and extravasation of leukocytes and erythrocytes into the interstitial space (Figure
24D). TNF/zVAD treatment induced endothelial detachment with evidence of leukocyte adherence and translocation in all 3 WT mice examined, whereas these morphological changes were observed in 2 of 4 WT mice treated with TNF. These histopathologic findings were not evident in TNF- or TNF/zVAD-treated Ripk1<sup>D138N/D138N</sup> mice, where liver endothelial barrier integrity was maintained similarly to untreated mice (Figure 24D).

Due to a lack of reliable measures of necroptotic death in mice, we isolated primary liver endothelial cells from TNF- and TNF/zVAD-treated WT and Ripk1<sup>D138N/D138N</sup> mice (80% average purity, Figure 27, A and B) and assessed MLKL activation. We consistently detected phospho-MLKL reactivity in endothelial cells isolated from TNF/zVAD-treated WT mice, but not in endothelial cells isolated from Ripk1<sup>D138N/D138N</sup> mice (Figure 24E). We were unable to detect phospho-MLKL reactivity in endothelial cells isolated from TNF-treated mice (Figure 28A), where endothelial breaks were observed only in half the mice examined (Figure 24D). Although unclear in the TNF shock model, TNF/zVAD-induced shock is mediated by RIPK1-dependent liver endothelial cell necroptosis.
Figure 27. Gating strategy and purity of endothelial cells isolated from TNF- or TNF/zVAD-treated WT and Ripk1<sup>D138N/D138N</sup> mice. (A) Gating strategy for the purification of liver endothelial cells using FACS. CD45+ cells were depleted before enriching for CD146+ cells and gating on CD45- CD31+ cells. (B) Quantification of liver endothelial cell enrichment from untreated (Unt.), or TNF or TNF/zVAD-treated WT and Ripk1<sup>D138N/D138N</sup> mice (n = 3). Error bars represent mean ± SEM.
Figure 28. Phosphorylated MLKL is detected in liver endothelial cells from TNF/zVAD-treated but not TNF-treated mice, nor is cleaved caspase-3 detected in TNF-treated livers. (A) WT and Ripk1^{D138N/D138N} endothelial cells were isolated from livers of untreated, TNF-, or TNF/zVAD-treated mice. A representative western blot showing protein lysates probed with antibodies to phospho-MLKL, MLKL, ERK1/2 is depicted. Lysates from murine embryonic fibroblasts (MEF) left untreated (Unt.) or stimulated with TNF, SMAC mimetics, and zVAD-fmk (T/S/Z) served as a positive control. (Lysates were generated and analyzed from 7 WT and 7 Ripk1^{D138N/D138N} TNF-treated mice). (B) Lysates from WT or Ripk1^{D138N/D138N} liver endothelial cells isolated from untreated or TNF-treated mice were probed with antibodies to full length and cleaved caspase-3 (CC3) or ERK1/2 as a loading control. MEFs left untreated (Unt.) or treated with TNF and cycloheximide (T/Cx) served as controls for CC3 detection. (C) Quantification of cleaved caspase-3-positive liver endothelial cells from untreated or TNF-treated WT and Ripk1^{D138N/D138N} mice by IHC (n = 3-6 mice per group). Error bars represent mean ± SEM.
Discussion

Here we demonstrate that the resistance of RIPK1 kinase inactive mice to TNF- and TNF/zVAD-induced shock is not due to hematopoietic cells and/or "cytokine storm", but rather due to lack of endothelial cell necroptosis, which prevents breaks in the vascular endothelium, activation of the clotting cascade and organ damage. Our results provide genetic evidence that RIPK1 kinase activity mediates liver endothelial cell necroptosis during TNF/zVAD-induced shock.

Cytokines such as IL-1β and IL-17α, and both apoptotic and necroptotic cell death, have been implicated in TNF-induced lethality. As reported previously in Nec-1 treated WT mice and Ripk3−/− mice, we found cytokine/chemokine induction to be normal in TNF-treated Ripk1D138N/D138N mice, however these mice failed to sustain cytokine levels in response to TNF administration. Interestingly, we show that TNF/zVAD-induced shock augmented the proinflammatory response in WT but not Ripk1D138N/D138N mice (Figure 15B), supporting the notion that necroptosis amplifies proinflammatory signaling and exacerbates organ injury. Multiple studies have shown that RIPK3 deficiency or pharmacologic inhibition of RIPK1 kinase activity decreases serum levels of DAMPs such as mitochondrial DNA (mtDNA) or markers of organ injury such as creatinine kinase, aspartate aminotransferase, or lactate dehydrogenase. Furthermore, DAMPs such as HMGB1 and extracellular histones are elevated in the blood of SIRS and sepsis patients, where they have been thought to result in chronic TLR activation and tissue injury.
TNF/zVAD administration elevated IL-1β levels, which were not systemically increased with TNF treatment alone. Caspase-1 and caspase-1/11-deficient mice, but not IL-1β/18-deficient mice, are susceptible to TNF-induced shock, thereby excluding a primary role for caspase-1-mediated inflammasome activation in TNF shock\textsuperscript{97,321}. Two recent reports have implicated MLKL activation in initiating inflammasome-mediated processing and release of IL-1β\textsuperscript{327,328}, suggesting that necroptosis sensitization in TNF/zVAD-induced shock and MLKL activation may contribute to increases in plasma IL-1β levels.

In addition to cell death signaling, RIPK1 kinase activity has been implicated in cytokine/chemokine production \textit{in vitro}\textsuperscript{103,277,278}, including reduced transcription and production of CXCL1 in TNF/zVAD-treated kinase inactive \textit{Ripk1}\textsubscript{K45A/K45A} BMDMs\textsuperscript{24}. In agreement with these recent reports, we find CXCL1, CXCL2 and IL-6 transcription and secretion impaired \textit{in vitro} in TNF/zVAD-treated \textit{Ripk1}\textsubscript{D138N/D138N} BMDMs (Figure 16), suggesting that reductions in the cytokine response in RIPK1 kinase inactive mice are due to effects of RIPK1 kinase activity on cytokine production rather than necroptotic cell death. With the exception of CXCL2, however, cytokine/chemokine production at early time points is unaffected by a lack of RIPK1 kinase activity (Figures 15 and 20), demonstrating that \textit{in vivo}, RIPK1 kinase activity is not required for cytokine/chemokine induction in response to a high dose of TNF. Significant reductions in plasma cytokine/chemokine levels were observed in TNF-treated \textit{Ripk1}\textsubscript{D138N/D138N} mice at 7h, consistent with the proinflammatory nature of necroptotic death. Additionally, \textit{Ripk1}\textsubscript{D138N/D138N} \textrightarrow\textit{WT}
chimeras had elevated cytokine/chemokine levels in response to TNF/zVAD administration (Figure 21), suggesting that RIPK1 kinase activity does not regulate cytokine/chemokine production when caspases are inhibited in vivo.

We found that 3 of 11 Ripk1\textsuperscript{D138N/D138N} → WT chimeras recovered from TNF/zVAD administration (Figure 18E), indicating that Ripk1\textsuperscript{D138N/D138N} hematopoietic cells can contribute to protection. Our data suggests that optimal cytokine/chemokine induction requires necroptosis competent (WT) hematopoietic and endothelial cells (Figure 20A), leading us to speculate that DAMPs released by necroptotic endothelial cells feedback on WT hematopoietic cells to induce necroptosis and further amplify cytokine levels. Thus, Ripk1\textsuperscript{D138N/D138N} hematopoietic cells may mediate recovery in Ripk1\textsuperscript{D138N/D138N} → WT chimeras by failing to undergo necroptosis in response to DAMPs released by WT necroptotic endothelial cells. Alternatively, recovery may be mediated by the survival of Ripk1\textsuperscript{D138N/D138N} hematopoietic cells and production of anti-inflammatory cytokines which support barrier repair.

A caveat of our bone marrow chimera studies is that protected WT → Ripk1\textsuperscript{D138N/D138N} mice retain radiation-resistant, tissue resident Ripk1\textsuperscript{D138N/D138N} macrophages such as Kupffer cells (KC) in the liver. KC associate with endothelial cells in the liver sinusoids and function to maintain liver homeostasis by surveying microbial products from the intestine. KC express TLRs and produce cytokines and chemokines in response to DAMPs or pathogen associated molecular patterns (PAMPs). Moreover, KC undergo necroptosis upon Listeria monocytogenes
infection. Thus, Ripk1\textsuperscript{D138N/D138N} KC may contribute to resistance to shock by failing to undergo necroptosis and/or produce cytokine/chemokines in response to microbial products or DAMPs released by necroptotic endothelial cells.

Although we detect decreases in cleaved caspase-3 positive ileal but not colonic IECs in TNF-treated Ripk1\textsuperscript{D138N/D138N} mice, no significant differences in intestinal apoptosis were observed between TNF/zVAD-treated WT or Ripk1\textsuperscript{D138N/D138N} mice (Figure 23). Consistent with these data, executioner caspases -3 or -7 and TNFR1-mediated IEC apoptosis do not appear to contribute to TNF-induced lethality. However, Newton et al. show that while there is evidence of slight hypothermia in Ripk3\textsuperscript{-/-} and Mlkl\textsuperscript{-/-} mice in response to TNF, Ripk3\textsuperscript{-/-} Caspase-8\textsuperscript{-/-} and Mlkl\textsuperscript{-/-} Caspase-8\textsuperscript{-/-} double-knockout mice are completely protected from TNF-induced hypothermia and have significantly reduced serum cytokine and chemokine levels. Additionally, they show that Caspase-8\textsuperscript{-/-} and Villin-cre Ripk3\textsuperscript{fl/fl} mice are largely protected from TNF-induced cytokine/chemokine expression and hypothermia, suggesting that in addition to necroptosis, caspase-8 contributes to lethality during TNF-induced shock. Since our data and others indicate no role for the caspase-1-mediated inflammasome or intestinal apoptosis in TNF-induced lethality, caspase-8 mediated effects likely reflect its role(s) in inflammasome-independent IL-1\beta regulation and processing.

Although TNF shock is typically thought of as a sterile model of injury, several reports show a key role for intestinal barrier dysfunction and systemic
spread of bacteria in the blood of TNF-treated mice\textsuperscript{312,313}. While not addressed in the Newton study, the Villin-cre Ripk\textsuperscript{3\textsubscript{fl/fl}} mice may be protected from hypothermia and increased cytokine levels because RIPK3-deficient IECs fail to undergo necroptosis and consequently the intestinal epithelial barrier is maintained. We show that Ripk\textsuperscript{1\textsubscript{D138N/D138N}} mice do not exhibit TNF-induced increases in intestinal permeability, nor do we detect spread of bacteria to peripheral organs such as the liver or spleen. While breaches in the intestinal epithelium and entry of bacteria or bacterial products into the bloodstream may contribute to TNF-induced shock, our data demonstrate that the downstream effects of the host response converge on the vascular endothelium.

We find that TNF-resistant Ripk\textsuperscript{1\textsubscript{D138N/D138N}} mice and WT $\rightarrow$ Ripk\textsuperscript{1\textsubscript{D138N/D138N}} chimeras do not exhibit increases in lung and liver vascular permeability, or evidence of increased tissue factor expression and coagulation. Furthermore, in the TNF/zVAD model, vascular barrier protection and an absence of coagulation, at least in the liver, correlate with an inability to detect phospho-MLKL reactivity in the protected RIPK1 kinase inactive endothelium. Our results indicate that necroptosis contributes to barrier disruption and activation of the clotting cascade, as we find MLKL activation in liver endothelial cells isolated from TNF/zVAD-treated WT mice.

Although TNF and TNF/zVAD treatment increase plasma tissue factor levels (Figure 22D) and induce coagulation (Figure 24, A and B), we detect fewer breaks in the liver endothelium of TNF-treated WT mice (Figure 24D). Consistent
with this observation, we were unable to detect phospho-MLKL reactive endothelial cells in livers of TNF-treated mice (Figure 28A). We hypothesized that TNF may stimulate RIPK1-kinase-dependent apoptosis, however, cleaved caspase-3 reactive cells were not detected in TNF-treated liver sections by immunohistochemistry or by immunoblotting endothelial cell lysates from TNF-treated mice (Figure 28, B and C), suggesting that apoptosis is not responsible for the vascular breaks. In contrast to TNF/zVAD administration where hypothermia and lethality is rapid and fully penetrant in WT controls (Figures 8, 9, and 18), mice treated with TNF take longer to exhibit symptoms (hypothermia, inactivity, hunched posture) and are variable in their presentation at sacrifice, 8 hours post TNF administration. We speculate that our inability to detect phospho-MLKL-reactive endothelial cells in mice treated with TNF reflects this variability in presentation and consequently fewer endothelial breaks (Figure 24D) and potentially less endothelial cell necroptosis was induced following TNF treatment for 8 hours compared to TNF/zVAD treatment for 4 hours.

We also demonstrate that lung endothelial cells isolated from WT but not Ripk1D138N/D138N mice undergo necroptosis in vitro when treated with TNF, Smac and zVAD, but not with TNF/zVAD only (Figure 25C). This finding was unexpected as TNF/zVAD administration is sufficient to induce liver endothelial cell necroptosis in vivo (Figure 24E). The requirement for Smac mimetic to induce endothelial necroptosis in vitro may reflect increased cellular pools of cIAP1/2 or XIAP in lung
versus liver endothelial cells or simply indicate that TNF/zVAD concentrations achieved in vivo are not accurately modeled in vitro.

Many septic patients have impaired endothelial barrier function, exhibit edema, coagulation abnormalities, and decreased levels or dysfunctional activation of anticoagulants. The most promising clinical trials have focused on combining anti-inflammatory and anticoagulant agents, such as activated protein C or thrombomodulin\textsuperscript{331}. Recent advances focus on methods to strengthen the vascular endothelium and modulate its response to proinflammatory cytokine levels. In several models of LPS endotoxemia or cecal ligation and puncture (CLP), enhanced vascular stability dramatically increased mouse survival, even though no reductions in the proinflammatory cytokine response were observed\textsuperscript{332,333}. Similarly, we demonstrate that chimeric mice susceptible to TNF-induced shock exhibit increased vascular permeability and coagulation even when proinflammatory cytokine levels are not sustained (Figure 20A, and Figure 22, E and F). We provide evidence that \textit{Ripk}\textsubscript{1}D\textsubscript{138N}/D\textsubscript{138N} liver endothelial cells fail to exhibit MLKL activation and undergo necroptosis (Figure 24E), thereby maintaining an intact vasculature that protects RIPK1 kinase inactive mice from TNF/zVAD-induced lethality. Interestingly, RIPK3 plasma levels are significantly increased in non-survivors of sepsis\textsuperscript{334}, suggesting that endothelial cell necroptosis may contribute to lethality in both SIRS and sepsis patients. \textit{Ripk}\textsubscript{1}D\textsubscript{138N}/D\textsubscript{138N}, \textit{Ripk}3\textsuperscript{-/-} and \textit{Mlk1}\textsuperscript{-/-} mice have been examined in mouse models of sepsis and although the results generated using the cecal ligation and puncture
model are conflicting\textsuperscript{48, 97, 335}, we and others find Ripk\textsuperscript{1D138N/D138N} mice susceptible to LPS administration (\textsuperscript{227}, and Figure 11), indicating that necroptosis may not be a critical factor in sepsis. The role of necroptosis in shock and sepsis needs to be explored further, but our results demonstrate that RIPK1 kinase inhibitors may have promise in ameliorating or preventing inflammatory vascular injury and SIRS.
Methods

Mice

*Ripk1<sup>D138N/D138N</sup>* mice were described previously<sup>40</sup>. For bone marrow transplantation studies, recipient C57BL/6 CD45.1 or CD45.2 (WT or *Ripk1<sup>D138N/D138N</sup>* mice received 11 Gy of total body irradiation in a split dose (550 rads) using a Cesium-137 irradiator, with a 4-hour rest between doses. Irradiated recipients were reconstituted by intravenous injection of 2x10<sup>6</sup> WT (CD45.1 or CD45.2) or *Ripk1<sup>D138N/D138N</sup>* (CD45.2) bone marrow cells. Recipients were maintained on medicated water for one month. Cheek bleeds were performed after 4 and 8 weeks to assess reconstitution efficiency by flow cytometry.

TNF-induced shock experiments

Male WT and *Ripk1<sup>D138N/D138N</sup>* littermates were injected intravenously with 9 μg mTNFα (Cell Sciences) only or in combination with 16.7 mg/kg zVAD-fmk (Bachem), unless noted otherwise. Body temperature and survival were monitored every few hours. Mice were used between 8 and 12 weeks of age. For ex vivo analyses mice were typically sacrificed after 4 (TNF/zVAD) or 8 hours (TNF). Reconstituted mice were injected with mTNFα and zVAD-fmk 2 months after reconstitution. When littermate controls were not used, sex- and age-matched mice were co-housed.
Cytokine Analysis

Plasma cytokines were measured using a 12-plex protein/peptide multiplex analysis (Luminex Technology) conducted by the National Mouse Metabolic Phenotyping Center at the University of Massachusetts Medical School. Cytokines and chemokines that were below the limit of detection were assigned a value of zero. Blood was collected via cardiac puncture in a heparinized needle and spun down to isolate plasma. The samples were stored at -80°C until analysis. Plasma tissue factor levels were determined by ELISA (R&D Systems). IL-6 (eBioscience), CXCL1, CXCL2, and CCL5 (R&D Systems) secretion by cultured primary cells was determined by ELISA.

Gene expression Analysis

Primary BMDMs were treated with 50 ng/mL TNF (R&D Systems) or TNF and 50 μM zVAD (Enzo) for 2 or 6 hours. Primary lung endothelial cells were treated with TNF (100 ng/mL) for 1 or 4 hours. Total cellular RNA was isolated using an RNeasy Mini Kit (QIAGEN). Total lung or liver RNA was extracted with TRIzol (Life Technologies). cDNA was prepared using the Superscript First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and run on the AB7300 Detection System (Applied Biosystems). Relative gene expression was normalized to Actin and determined using the ΔΔCT method, with the following primer sequences:
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Direction</th>
<th>Primer sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>For</td>
<td>CGAGGCCAGAGCAAGAGAG</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>CGGTTGGCCTTAGGGTTTCA</td>
</tr>
<tr>
<td>E-selectin</td>
<td>For</td>
<td>ATGCCCTCGGCTTTCTCTCTCT</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>GTAGTCGGCTGACAGTATGC</td>
</tr>
<tr>
<td>P-selectin</td>
<td>For</td>
<td>CATCTGGTTCACTGTTTTGATCT</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>ACCCGTGAGTTATCCATGAGT</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>For</td>
<td>GTGATGCTCAGGTATCCATCCA</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>CACAGTTCTCAAAGCAGCG</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>For</td>
<td>AGTTGGGATTCGGTTTGGTCT</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>CCCCTCATTCTTACCACCC</td>
</tr>
<tr>
<td>Il6</td>
<td>For</td>
<td>AACGATGATGCACCTGCAGA</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>GAGCATGGGAAATGGGTTA</td>
</tr>
<tr>
<td>Ccl5</td>
<td>For</td>
<td>GCCCCACGTCAGGAGATTTTCTA</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>ACACACTTGGCGGTCTCC</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>For</td>
<td>CTGGGATTCCCTCAAGAACATC</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>CAGGGTCAAGGGCAAGCCTC</td>
</tr>
<tr>
<td>Cxcl2</td>
<td>For</td>
<td>CCAACCACCGGCTACAGG</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>GCGTCACACTCAAGCTCTG</td>
</tr>
</tbody>
</table>

Flow Cytometry

Single-cell suspensions from blood, spleen, bone marrow, lung and liver were stained with cell-surface antibodies for myeloid (Gr-1, CD11b, CD11c, and Ly-6C), lymphoid (CD4, CD8, and B220) and endothelial (CD31) markers. To distinguish between donor and host hematopoietic cells in the bone marrow reconstitution experiments, anti-CD45.1 and anti-CD45.2 antibodies were used. DAPI (Molecular Probes) was used to distinguish between live and dead cells. All samples were run on a BD LSRII flow cytometer (BD Bioscience) and analyzed using FlowJo software (Tree Star) with the following antibodies:
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Fluorophore</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>FITC</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Gr-1</td>
<td>RB6-8C5</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Ly-6C</td>
<td>HK1.4</td>
<td>APC</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Cd11c</td>
<td>N418</td>
<td>Pacific Blue</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD45.1</td>
<td>A20</td>
<td>PerCP/Cy5.5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD45.2</td>
<td>104</td>
<td>PeCy7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD4</td>
<td>RM4-5</td>
<td>PerCP/Cy5.5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD8</td>
<td>53-6.7</td>
<td>FITC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>B220</td>
<td>RA3-6B2</td>
<td>PE</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD45</td>
<td>30-F11</td>
<td>APC-Cy7</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD31</td>
<td>390</td>
<td>FITC</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>

Prothrombin Time Test

Blood was collected via cardiac puncture and mixed with 3.2% sodium citrate in a 9:1 ratio. Plasma was isolated and the test was conducted by IDEXX (Grafton, MA).

Vascular Permeability Assay

A 1% Evans Blue (Sigma) solution was injected iv at 40 mg/kg six hours after mTNF-α administration. After 1 hour the mice were sacrificed, perfused and organs were dried overnight before homogenization in formamide (Sigma). The amount of extracted Evans Blue dye was assessed by spectrophotometry at 620nm on a Beckman Coulter DTX880 plate reader.
**Intestinal Permeability Assay**

Mice were orally gavaged with 100 μL of 150 mg/kg Fluorescein isothiocyanate-dextran (M.W. 4000) beads (Sigma) 2 hours after TNF administration. 6 hours later blood was drawn via cardiac puncture. The amount of FITC-dextran in plasma was measured on a Beckman Coulter DTX880 plate reader (excitation wavelength: 488 nm, emission wavelength: 530 nm).

**Histology and Immunohistochemistry**

Livers were fixed in 2% paraformaldehyde (Fisher Scientific). Samples were embedded in paraffin and H&E staining was done on 8 μm thick sections according to standard protocols. Cleaved caspase-3 staining was done on 6-micron thick sections at 1:100 dilution of cleaved caspase-3 antibody (cat # 9661, Cell Signaling) according to standard protocols. For endomucin staining, 3 μm thick liver sections were processed through xylene and rehydrated through an ethanol gradient prior to immersion in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6) and antigen retrieval was done using a 2100 Antigen retriever. Sections were incubated for 1hr at room temperature (RT) in blocking solution (1% rabbit serum, 1% BSA, 0.3% Triton-X 100 in PBS), and then kept in primary antibody mix (Endomucin, 1:200, Santa Cruz sc-65495; in 2.5% rabbit serum in PBS) overnight at 4°C. Rat secondary antibody was used for 1 hour at room temperature (Vector biotinylated anti-rat IgG). Sections were developed using Vectastain Elite ABC Kit and DAB Peroxidase Substrate Kit according to manufacturer’s instructions (Vector Laboratories) before counterstaining with
Hematoxylin and mounting with Vectashield dry mounting media. Images were acquired using a Nikon Eclipse 80i microscope. To quantify the percentage of clotting in liver blood vessels, 8 random fields at 20x field of view were counted per mouse. To quantify the percentage of cleaved caspase-3 positive cells in the intestines, 10 random fields at 40x field of view were counted per mouse. To quantify the number of cleaved caspase-3 positive cells in the intestines or liver, 10 random fields at 40x field of view were counted per mouse.

**Cell culture**

BMDMs were generated by culturing bone marrow cell suspensions in 20% conditioned media from L929 cells for seven days. On day 7 adherent cells were re-plated in 6-well plates for mRNA experiments (2x10^6 cells per well) or in 96-well plates for ELISA assays (2x10^5 cells per well). Mouse lungs were digested in 25 mL of 1 mg/mL collagenase at 37°C for an hour. After sequential filtering through a 100 μm and 40 μm cell strainer, the cell suspension was plated on a gelatin-coated T75 flask and the cells were incubated for 4-6 days until reaching confluence. Cells were grown in endothelial cell media containing low glucose DMEM/F-12 with 20% FBS, 1% L-glutamine, 1% Pen-Strep, 100 μg/mL Heparin and 50 μg/mL endothelial cell growth supplement (Biomedical Technologies Inc.).

To immunoselect endothelial cells, confluent T75 flasks were incubated with CD-31 (PECAM-1, cat # 553369, BD Biosciences) and CD102-coated (ICAM-2, cat # 553325, BD Biosciences) Dynabeads on ice for 20 minutes. Using a magnetic
separator, bead-bound cells were recovered and washed 3 times with PBS. Endothelial cells were plated on gelatin-coated T25 flasks and expanded.

Liver endothelial cell isolation

Mice were perfused with PBS, livers were minced with a razor blade on ice, and shaken for 30 minutes at 37°C in 1 mg/mL collagenase. The suspension was filtered through a 100 μm strainer and spun for 5 minutes at 50g to separate out hepatocytes. The supernatant was spun again for 5 minutes at 60g before the non-parenchymal cell fraction in the supernatant was pelleted at 700g for 10 minutes. After red blood cell lysis, the non-parenchymal liver cell fraction was depleted of CD45+ positive cells before enriching for CD146+ endothelial cells with MACS MicroBeads, according to manufacturer’s instructions (Miltenyi Biotec). Purity was checked by FACS and recovered cells were snap frozen to use for Western blot.

Immunoblotting

Isolated liver endothelial cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% NP40, 0.25% deoxycholate, 0.1% SDS, 1 mM EDTA), supplemented with protease inhibitors (Roche Applied Science), supplemented with sodium orthovanadate (1 mM), phenylmethane-sulfonyl fluoride (PMSF, 1 mM), and dithiothreitol (DTT, 1 mM). Total protein was run on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane (Invitrogen) and probed with antibodies to phospho-MLKL (ab196436, Abcam), MLKL (gift from Jiahuai Han, and AP14272b, Abgent), caspase-3 (cat # 9662, Cell Signaling), and ERK1/2 (cat #
9102, Cell Signaling). Membranes were developed with Clarity Western ECL Substrate (BioRad).

**Cell viability**

For apoptosis and necroptosis assays, cultured lung endothelial cells were pre-treated with 1 μM Smac mimetic (ChemieTek) or 20 μM zVAD-fmk (Enzo) prior to adding TNF (10 ng/mL). Metabolic activity was assayed after 20 hours by addition of MTS reagent (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega) and measured using a Beckman Coulter DTX880 plate reader. Absorbance values at 535nm were normalized to Smac mimetic control.
CHAPTER V

Discussion
This thesis research has focused on elucidating the role of RIPK1 kinase activity in regulating pro-inflammatory and pro-death signaling in response to TNFR1 or TLR3/4 activation. I demonstrate that the kinase activity of RIPK1 contributes to necrosome formation and regulates both apoptosis and necroptosis induction in response to certain stimuli in MEFs and BMDMs. I have shown that RIPK1 kinase activity regulates transcriptional and/or translational activation of TNF/zVAD-induced cytokines and chemokines in vitro, but does not play a role in their regulation in vivo. Additionally, the lack of RIPK1 kinase activity mediates protection from TNF- and TNF/zVAD-induced shock by critically acting in non-hematopoietic lineages, protecting the intestinal epithelial and vascular endothelial barriers from permeability increases and clotting, in part by abrogating endothelial cell necroptosis. Thus, this work disproves the cytokine storm idea and helps clarify the role of RIPK1 kinase activity in driving TNF-induced inflammation and shock in mice via necroptosis and effects on the endothelium.

Early studies of the serine/threonine kinase RIPK1 identified its prominent role in proinflammatory and cell survival signaling downstream of TNFR1 and TRIF-dependent TLRs 3/4 by acting as a scaffold to mediate optimal NF-κB and MAP kinase pathway activation. Though these functions were kinase-independent, RIPK1 was found to have additional kinase-dependent death roles, as it could induce caspase-8 dependent apoptosis or caspase-independent RIPK1/RIPK3/MLKL-mediated necroptosis. However, most of these studies were done with in vitro reconstitution of kinase inactive RIPK1 mutants, or utilizing the
original, less specific and less potent, Nec-1 inhibitor\textsuperscript{289}. Especially \textit{in vivo}, the role of RIPK1 kinase activity was unclear as Nec-1 was determined to either sensitize or protect mice in an inflammatory TNF-induced shock model\textsuperscript{97–99}. To determine the role of RIPK1 kinase activity in mediating inflammation and necroptosis \textit{in vivo}, we generated kinase inactive \textit{Ripk1}^{D138N/D138N} mice and examined their responses to pro-apoptotic and pro-necroptotic stimuli \textit{in vitro} and \textit{in vivo}.

Our work in chapter 2 demonstrated that the kinase activity of RIPK1 is required for efficient necrosome formation with RIPK3, and for mediating TNF/Cx/zVAD or TNF/Smac/zVAD-induced necroptosis in MEFs. Previous studies have suggested that RIPK3 is recruited into the initial pro-apoptotic complex IIb or Ripoptosome to form the necrosome\textsuperscript{43,44,161,162}. However, it remains unclear whether when pulling down the complex with caspase-8 or RIPK3 antibodies we detect the same cytosolic complex, or different cytosolic pools of these interacting necrosome components. RIPK1 and RIPK3 are reported to oligomerize and form amyloid fibrils upon necroptotic stimulation\textsuperscript{201}, and indeed in instances of complex formation we consistently detected decreased levels of the complex components caspase-8, RIPK1, and RIPK3 in the whole cell lysate. This suggests that the necrosome is indeed oligomerizing and we are not solubilizing it efficiently with our Triton X-100 detergent used in the endogenous lysis buffer. Several studies have shown that the RIPK1/RIPK3 necosome preferentially localizes to a Triton/NP-40-insoluble, SDS- or urea-soluble fraction \textit{in vitro} and \textit{ex vivo}\textsuperscript{203,294}. Thus, varying the detergents in our immunoprecipitations or lysing the insoluble fraction with a
harsher detergent may reveal additional qualitative or quantitative differences in the caspase-8/RIPK1/RIPK3 necrosome complex.

Interestingly, our data show that RIPK3 kinase activity is not necessary for RIPK3 recruitment or for stable formation of a TNF/Smac/zVAD-induced necrosome complex that includes caspase-8 and presumably FADD (Figure 5). While RIPK3 kinase activity was not required for stable complex formation, it was required to induce necroptosis in TNF/Smac/zVAD-stimulated MEFs. A similar scenario has been reported in TNF/Cx/zVAD-stimulated MEFs\(^3\). While Nec-1 blocked necrosome assembly, the RIPK3 kinase inhibitors did not affect necrosome assembly but were required for the induction of necroptotic death\(^3\). Thus, in TNF/Cx/zVAD- or TNF/Smac/zVAD-treated MEFs, necrosome formation requires the kinase activity of RIPK1 but not RIPK3, although the kinase activities of both are required for necroptosis to ensue\(^3\). These results suggest that RIPK1 kinase activation and conformational change allows it to auto-phosphorylate and recruit RIPK3 via RHIM-mediated interactions to the necrosome. Although RIPK3 can phosphorylate RIPK1 \textit{in vitro}\(^4\,^8\), this trans-phosphorylation event does not seem necessary for stable RIPK1/RIPK3 interaction in this context. Use of the RIPK3 kinase inhibitors, instead of a kinase inactive RIPK3, may cause conformational changes that promote the interaction of RIPK3 with RIPK1, FADD, and caspase-8\(^2\,^2\). Additional experiments interrogating the role of RHIM mutant and kinase inactive RIPK1 and RIPK3 in mediating stable necrosome formation in MEFs would be informative to elucidating the importance of RIPK1 and RIPK3 in...
recruitment and auto- and trans-phosphorylation of the necosome components.

Utilizing RIPK1 kinase inactive mice, we were able to definitively prove that RIPK3 and RIPK1 kinase activity play crucial roles in mediating TNF- and TNF/zVAD-induced shock or SIRS (Figures 8 and 9), in agreement with Duprez et al.\textsuperscript{97} Similarly, RIPK1 kinase inactive mice seemed partially protected from lower doses of LPS-induced hypothermia and lethality, in line with the prominent LPS-induced TNF upregulation that occurs \textit{in vivo} (Figure 12). However, a higher LPS dose was not protective and importantly, RIPK1 kinase inactive littermates were sensitized to LPS-induced hypothermia. Thus, using littermate controls in these shock studies is critically important as microbiota differences exist even between age-, sex- and strain-matched non-littermate mice\textsuperscript{302}. Recently, several groups identified caspase-11 as sensing cytoplasmic LPS and activating a non-canonical inflammasome, mediating pyroptosis and IL-1\(\beta\)/IL-18 release. Indeed, caspase-11-deficient and IL-1\(\beta\)/IL-18 double knock-out mice were largely resistant to LPS-induced shock\textsuperscript{321,336–338}. Our LPS shock data suggest that RIPK1 kinase activity does not play a role in this caspase-11-dependent pathway, consistent with a recent report that \textit{Ripk1}\textsuperscript{D138N/D138N} and \textit{Ripk3}\textsuperscript{-/-} littermates are susceptible to LPS but not TNF-induced shock\textsuperscript{227}. Interestingly, RIPK1 K45A kinase inactive mice are partially, but significantly, protected from high dose LPS-induced lethality\textsuperscript{278}, suggesting that specific RIPK1 mutations and potential conformational changes, or microbiota differences in mice, may reveal some dependence of LPS-induced shock on RIPK1 kinase activity.
While our work implicates MLKL activation and endothelial necroptosis in TNF/zVAD-induced shock, a recent study has demonstrated that during LPS-induced shock caspase-11-mediated endothelial cell pyroptosis in lungs leads to acute lung injury, increases in lung vascular permeability, neutrophil infiltration, and death\textsuperscript{339}. Interestingly, both necroptosis and pyroptosis execution involves oligomerization and translocation of channel or pore-forming proteins to the plasma membrane. While MLKL oligomerization is activated by RIPK3-mediated phosphorylation, the pyroptosis executioner protein gasdermin-D is activated and oligomerizes upon caspase-1 or caspase-11-mediated proteolytic cleavage\textsuperscript{58–60}. This releases the N-terminal p30 fragment, typically in an autoinhibitory conformation with the C-terminal region, allowing it to oligomerize and translocate to the plasma membrane where, like MLKL, it binds phosphatidylinositol phosphates and forms pores\textsuperscript{340–343}. Interestingly, while MLKL is thought to form ion-selective channels leading to cation influx and swelling and bursting of the plasma membrane, N-terminal gasdermin-D fragment oligomerization seems to form non-selective ion-permeable pores\textsuperscript{343}. Plasma membrane rupture during pyroptosis is not accompanied by swelling and the cells produce pyroptotic bodies prior to rupture\textsuperscript{343}. Thus, while multiple forms of regulated necrosis may affect endothelial cell function and/or viability and contribute to lethality in mouse models of TNF- or LPS-induced shock, the differential effects of their plasma membrane rupture \emph{in vivo} remain to be elucidated further.

RIPK1 kinase activity has been implicated in LPS-induced inflammatory
gene regulation *in vitro* and *in vivo*, mediated in part by RIPK1 and RIPK3 kinase-dependent regulation of ERK, cFos, NF-κB, and STAT1 activation\(^{277,278}\). We observed diminished upregulation of TNF and IL-1β levels in the blood of non-littermate RIPK1 D138N kinase inactive mice treated with 5mg/kg LPS (Figure 12), similar to a report in RIPK1 K45A mice\(^{278}\). More fine-tuned regulation by RIPK1 D138N kinase activity specifically in bone marrow CD11b+ macrophages was demonstrated with a sub-lethal LPS dose, which revealed reduced transcription and secretion of LPS-induced TNF, CCL3, CCL4, GM-CSF, and IL-6 in RIPK1 kinase inactive mice\(^{277}\). We could confirm an LPS/zVAD- but not LPS-induced transcriptional regulation of *Tnf* and *Csf2* in *Ripk1\(^{D138N/D138N}\*) BMDMs, but no clear effect on TNF- or IL-1β induced secretion as reported in the literature\(^{277,278}\). Additionally, *in vivo* differences in RIPK1 kinase-dependent cytokine/chemokine levels were observed with LPS stimulation alone, whereas *in vitro* regulation depended on caspase inhibition with zVAD in our work and the Najjar et al., but not Shutinoski et al. studies\(^{277,278}\). These discrepancies need to be addressed with additional experiments but may reflect inherent differences in conformational changes of RIPK1 or degree of kinase inhibition of the D138N and K45A mutations. Additionally, caspase-8 activity may be different *in vitro* and *in vivo*, thereby explaining why caspase inhibition with zVAD is not necessary to reveal RIPK1 kinase-dependent regulation in mice\(^{277}\). In summary, our data suggests that RIPK1 kinase activity regulates LPS/zVAD-induced *Tnf* and *Csf2* upregulation *in vitro* and, at lower doses, LPS-induced cytokine levels *in vivo*. 
We found that TNF-induced upregulation of cytokine/chemokine levels in vivo did not depend on RIPK1 kinase activity for transient induction, but seemed required for sustained inflammation (Figure 15A). This suggests that at lethal TNF doses, RIPK1 kinase activity does not have a regulatory role in cytokine/chemokine production, but that sustained inflammation is reduced due to lack of necroptosis and DAMP release. Similar to the dichotomy between LPS and LPS/zVAD-induced responses in vitro and in vivo, we observed TNF/zVAD-induced RIPK1 kinase-dependent transcriptional and translational regulation of CXCL1, CXCL2, and IL-6 in primary BMDMs in vitro. Interestingly, CXCL2 levels were reduced in RIPK1 kinase inactive cells or mice in response to TNF alone, suggesting transcriptional regulation by a RIPK1 kinase-dependent mechanism that is unclear at this point. Our in vitro data supports a RIPK1 kinase-dependent role for TNF/zVAD-induced regulation of CXCL1 and demonstrates a novel role in IL-6 regulation, but could not confirm a role in CCL5 regulation. Since these in vitro effects appear to be death-independent, future studies will need to resolve discrepancies as to what specific cytokines/chemokines RIPK1 kinase activity regulates in a transcriptional or translational manner, and what the mechanism of this regulation is in BMDMs in vitro. Importantly, our in vivo data argues that RIPK1 kinase-dependent necroptosis, and not cytokine regulation, drives sustained inflammation via DAMP release.

Our bone marrow chimera studies demonstrate that RIPK1 kinase activity in non-hematopoietic lineage/s drives TNF- and TNF/zVAD-induced shock,
contrary to the expectation that hematopoietic cell-mediated cytokine storm is a main mediator of lethality\textsuperscript{303}. Indeed, we observed that TNF sensitive \textit{Ripk1}^{D138N/D138N} \rightarrow WT mice exhibit reduced proinflammatory cytokine/chemokine levels as the shock progresses. However, IL-17R-deficient and IL-1β/IL-18 double knock-out mice are protected from a lethal TNF and/or LPS-induced shock\textsuperscript{320,321}. Additionally, IL-1β and IL-17α levels are found increased in septic patients\textsuperscript{308,309} and were elevated systemically in WT mice treated with TNF/zVAD, but not TNF (Figure 15). Thus, although our data indicates that the cytokine storm may not be the main driver of lethality, the importance of certain cytokines/chemokines, such as IL-17α, IL-1β, and IL-18 should not be over-looked. Since we determined cytokine/chemokine levels systemically in the plasma, it remains possible that there are differences in the systemic versus local tissue or organ inflammatory responses in WT and RIPK1 kinase inactive mice. For instance, is IL-17α expression increased in the intestines of TNF-treated WT mice but not detected systemically\textsuperscript{320}?

The levels of various organ damage markers, DAMPs, and cytokines are reduced in TNF-treated RIPK3-deficient mice compared to WT controls\textsuperscript{97}. Meanwhile, we observed that kinase inactive RIPK1 mice are protected from TNF-induced intestinal epithelial and vascular endothelial permeability increases and tissue factor upregulation and clotting. We were able to show that resistant reconstituted WT \rightarrow \textit{Ripk1}^{D138N/D138N} mice are also protected from increases in tissue factor expression and vascular permeability. Although we detected evidence
of endothelial cell necroptosis in TNF/zVAD-treated mice via pMLKL activation, we could not detect endothelial cell apoptosis (cleaved caspase-3 positive cells) or necroptosis (pMLKL activation) in TNF-treated mice (Figure 28). Thus, while the TNF- and TNF/zVAD-mediated effects seem to converge on the endothelium, and necroptotic death of endothelial cells contributes to the induction of clotting, it is unclear what type(s) of cell death occur during TNF-induced shock. We could address this by looking at levels of organ damage markers and DAMPs (such as mitochondrial DNA) in TNF- and TNF/zVAD-treated non-reconstituted and reconstituted mice. Additionally, the development of a phospho-MLKL antibody suitable for flow cytometry assays or optimized for immunohistochemical staining will allow us to more definitively identify necroptotic cells. While we detected pMLKL reactivity in TNF/zVAD-treated endothelial cells, we could not detect evidence of MLKL activation in TNF- or TNF/zVAD-treated CD45+ hematopoietic cells isolated from the liver (data not shown). Thus, WT hematopoietic cells may not respond to DAMPs or undergo necroptosis during TNF-induced shock, while endothelial cells seem sensitized to necroptosis.

A recent study showed that necroptosis is less inflammatory in vitro and in vivo compared to TNF or LPS stimulation because necroptotic death of the cells terminates the potent TNF- or LPS-driven proinflammatory signaling pathways. While seemingly at odds with our conclusions, there are several differences between our studies that may explain these discrepancies. Kearney and colleagues inject supernatant from TNF- or TNF/zVAD-stimulated necroptotic
L929 cells into the peritoneal cavity of immunocompetent Balb/c mice and observe reduced infiltration of inflammatory cells in response to supernatant from necroptotic compared to TNF-stimulated cells. While necroptotic supernatant contained reduced levels of inflammatory cytokines and chemokines prior to injection (due to high levels of cell death), we see systemic as well as organ-specific increases in proinflammatory cytokines and the chemokines CXCL1 and CXCL2 with TNF/zVAD treatment via intravenous injection compared to TNF alone. Thus, in vivo, a systemic pro-necroptotic TNF/zVAD stimulus is more proinflammatory compared to TNF alone. The type and amount of DAMPs released by various cells in vivo or their modulation may be different than DAMPs released by a cell line in vitro. Additionally, our reconstitution data argues that non-hematopoietic lineages drive TNF- or TNF/zVAD-induced shock while cytokine/chemokine levels do not necessarily correlate with survival. This is supported by the fact that we demonstrate evidence of liver endothelial cell but not hematopoietic cell necroptosis via pMLKL activation. Thus, in our model, the hematopoietic cells which drive proinflammatory signaling do not seem to succumb to necroptosis, which could reduce their numbers and dampen the inflammatory response. Instead, necroptosis of non-hematopoietic lineages such as liver endothelial cells may potentiate inflammatory signaling by releasing DAMPs and sustaining the hematopoietic cell-driven proinflammatory response.

Although the factors dictating the type of cell death induced in vivo are not well understood, the activation status of caspase-8 and balance of anti-apoptotic
and pro-necroptotic proteins may dictate the decision. Expression differences in cIAP, cFLIP, RIPK3, and MLKL levels and the localized tissue context may sensitize certain cell types to apoptosis or necroptosis\textsuperscript{89}. \textit{Mlkl}\textsuperscript{-/-} mice are partially sensitive to TNF-induced hypothermia, suggesting that necroptosis contributes to, but is not the only cause of, TNF-induced shock\textsuperscript{227}. Meanwhile, intestinal epithelial cell-specific deletion of RIPK3 phenocopied the resistance of \textit{Ripk3}\textsuperscript{-/-} mice to TNF-induced shock, arguing for RIPK3-mediated signaling or death in the intestine as the determining factor\textsuperscript{227}. We found a slight but significant decrease in the amount of apoptosis induced in the ileum, but not colon, of TNF-treated \textit{Ripk1}\textsuperscript{D138N/D138N} mice compared to WT, but no TNF-induced intestinal permeability increase. While TNF did induce intestinal permeability increases in WT mice, we could not detect systemic evidence of bacteremia. These results suggest that TNF-induced apoptosis does not mediate increases in TNF-induced intestinal permeability\textsuperscript{97,313}. However, RIPK1 kinase-dependent signaling or apoptosis may mediate Paneth and/or goblet cell dysfunction or death in the intestine, leading to decreased expression of anti-microbial peptides and defensins, and a reduction in mucin levels\textsuperscript{172,313}. Bacterial translocation during TNF-induced shock could converge on the liver via the portal vein, with microbial products influencing endothelial cell dysfunction and death. Independently of their pro-death effects, RIPK1 kinase activity and RIPK3 have recently been shown to promote VEGF-induced vascular permeability via p38 and HSP27 signaling\textsuperscript{344}. Thus, RIPK1- and RIPK3-dependent
signaling and/or pro-death effects in intestinal epithelial and vascular endothelial cells may mediate TNF-induced shock.

In addition to TNF-induced effects on intestinal epithelial and vascular permeability and dysfunction, mice undergoing TNF-induced shock exhibit rapid drops in heart rate and mean arterial pressure, leading to hypotension. Interestingly, co-administration of zVAD with TNF sensitizes mice to these cardiovascular effects while inhibiting soluble guanylate cyclase with methylene blue prevents hemodynamic collapse and lethality. Additionally, recent literature has shown that stabilizing the vascular barrier is protective in various mouse models of shock and sepsis, even when proinflammatory signaling and the cytokine storm are not ameliorated. Thus, lethality during TNF- and TNF/zVAD-induced shock seems to be driven in large part by dysfunction or collapse of the cardiovascular system. Loss of blood pressure and edema due to increased vascular permeability contributes to clotting and multi-organ failure, ultimately resulting in death of the animal. While we observe evidence of liver endothelial necroptosis in WT but not RIPK1 kinase inactive TNF/zVAD-treated mice, RIPK1 kinase activity also leads to increases in intestinal epithelial and vascular endothelial permeability and clotting in response to either TNF or TNF/zVAD administration. While not addressed in this thesis, future studies should examine the role of RIPK1 kinase activity in mediating cardiac dysfunction and hypotension. Additionally, RIPK1 kinase activity may be required in signaling pathways mediating increases in epithelial and/or vascular permeability, or in
up-regulating tissue factor expression on hematopoietic and/or endothelial cells, thus contributing to the clotting observed in TNF- and TNF/zVAD-treated WT mice.

Some caveats and limitations of our study are that we do not know whether TNF- or TNF/zVAD-induced cytokines/chemokines (such as IFN-γ) converge on liver endothelial cells (or other cell types) to mediate dysfunction and/or cell death in a RIPK1 kinase-dependent manner. The kinetics of TNF/zVAD-induced shock are rapid and fully penetrant, whereas variations in SIRS symptoms are more pronounced during TNF-induced shock. It is plausible that this variability precluded us from detecting MLKL activation in TNF-treated mice and may reflect that less liver endothelial cell necroptosis is induced compared to TNF/zVAD-treated mice, consistent with the differences in kinetics. Additionally, even though neutrophil recruitment to the lung or liver is unaffected in resistant reconstituted mice, neutrophils can mediate damage through release of ROS and proteases. This could exacerbate cytokine production and inflammation, but RIPK1 kinase inactive cells may be resistant to these effects. Our bone marrow chimera data demonstrating protection from TNF- or TNF/zVAD-induced shock in Ripk1$^{D138N/D138N}$ hosts raise the issue of RIPK1 kinase inactive radiation-resistant tissue-resident macrophages, such as liver Kupffer cells (KC). KCs are present in the lumen of liver sinusoids and act as sensors of gut-derived bacteria and microbial products$^{346}$. Upon activation they release ROS and secrete cytokines and chemokines. During *Listeria monocytogenes* infection, KCs have been shown to undergo necroptosis$^{329}$. Thus, radiation-resistant Ripk1$^{D138N/D138N}$ KCs may not
secrete inflammatory cytokines or undergo necroptosis in response to microbial products or DAMPs released during shock. We will need to address this caveat by depleting macrophages with clodronate liposomes prior to testing the degree of resistance in TNF- and TNF/zVAD-treated WT \( \rightarrow Ripk1^{D138N/D138N} \) chimeras.

Future studies will need to further elucidate the role of RIPK1 phosphorylation in apoptotic and necrototic signaling to better understand its regulation. To date, the only known target of RIPK1 kinase activity is RIPK1 itself. Since RIPK1 can mediate both apoptosis and necroptosis in a kinase-dependent manner, what other complex components are phosphorylated during pro-death signaling? Alternatively, if RIPK1 indeed targets only itself for auto-phosphorylation, more clarity is needed to understand how regulation of its ubiquitination, phosphorylation, and conformational changes determine the signaling outcome\(^{171,241,242}\).

While we were able to show that liver endothelial cells undergo pMLKL activation and necroptosis during TNF/zVAD-induced shock \textit{in vivo}, genetic evidence suggests that both caspase-8 dependent activity and MLKL-dependent necroptosis contribute to TNF-induced shock\(^{227}\). A systematic study of caspase-8- and MLKL-dependent responses to TNF- and TNF/zVAD-induced shock would be useful to assess the contribution of apoptosis or necroptosis (or death-independent signaling effects) to epithelial and endothelial barrier permeability increases, clotting, cytokine/chemokine levels, hypothermia, and lethality. We attempted to assess the role of RIPK1 kinase activity in endothelial cells by utilizing tamoxifen-
inducible \textit{VeER}^{T\text{2}C\text{re}} \textit{Ripk1}^{D138N/FL} mice. However, the resistance of D138N heterozygous mice (\textit{Ripk1}^{D138N/+}) precluded us from this genetic approach (Figure 9). Thus, the generation of conditional RIPK1 D138N and conditional MLKL mice will allow investigation of organ- and cell type-specific induction of apoptosis and/or necroptosis in TNF- and TNF/zVAD-induced shock. For instance, determining the contribution of RIPK1 kinase activity specifically in intestinal epithelial cells or in the endothelium to TNF- and TNF/zVAD-induced inflammation, permeability, clotting, and death.

While our study revealed a crucial role for RIPK1 kinase activity in driving TNF- and TNF/zVAD-induced shock, the literature regarding the contribution of RIPK1, RIPK3, and MLKL to LPS-induced shock or the more relevant cecal ligation and puncture (CLP) sepsis model is unclear\textsuperscript{24,40,41,48,97,227,270,335}. Thus, it will be important to examine the involvement of RIPK1 kinase activity in LPS- versus LPS/zVAD-induced shock and in the polymicrobial sepsis (CLP) model. Additionally, RIPK3 conditional, RIPK3 kinase inactive, and MLKL-deficient mice should be tested in these models. These future studies will be crucial to further elucidating the exact role of RIPK1 kinase activity and pro-necroptotic regulators in various cell types during models of shock and sepsis. Work in this thesis presents evidence for a role of RIPK1 kinase-mediated endothelial cell necroptosis in the liver and direct or indirect effects on intestinal and vascular permeability and clotting during TNF- and TNF/zVAD-induced shock. Thus, RIPK1 kinase inhibitors
should be tested in patients as they may have promise in ameliorating inflammatory vascular injury and/or SIRS.
References


78. Varfolomeev EE, Boldin MP, Goncharov TM, Wallach D. A potential mechanism of "cross-talk" between the p55 tumor necrosis factor receptor and Fas/APO1: proteins binding to the death domains of the


Kanayama A, Seth RB, Sun L, et al. TAB2 and TAB3 Activate the NF-κB Pathway through Binding to Polyubiquitin Chains. Mol Cell.


137. Haas TL, Emmerich CH, Gerlach B, et al. Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex


180. Rickard JA, O'Donnell JA, Evans JM, et al. RIPK1 regulates RIPK3-MLKL-


Menon MB, Gropengießer J, Fischer J, et al. p38MAPK/MK2-dependent


253. Fitzgerald KA, Rowe DC, Barnes BJ, et al. LPS-TLR4 Signaling to IRF-3/7


282. Nogusa S, Thapa RJ, Dillon CP, et al. RIPK3 Activates Parallel Pathways of MLKL-Driven Necroptosis and FADD-Mediated Apoptosis to Protect


308. Sjövall F, Morota S, Frostner EÅ, Hansson MJ, Elmé E. Correction: Cytokine and Nitric Oxide Levels in Patients with Sepsis – Temporal


323. Ren Y, Su Y, Sun L, et al. Discovery of a Highly Potent, Selective, and


