RIPK3 activation: Necroptotic cell death and continued cytokine synthesis

Susana L. Orozco

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2018

Reading Committee:

Andrew Oberst, Chair

Keith Elkon

Adam Lacy-Hulbert

Program Authorized to Offer Degree:

Molecular and Cellular Biology

University of Washington

Abstract

RIPK3 activation: Necroptotic cell death and
continued cytokine synthesis

Susana L. Orozco

Chair of the Supervisory Committee:
Assistant Professor Andrew Oberst
Department of Immunology

Billions of cells die in our body each day, and our immune system is given the challenging task of determining which of these cell death incidents are normal, homeostatic events, and which are potentially dangerous. Necroptosis is a form of non-apoptotic programmed cell death mediated by the kinase RIPK3 and its downstream effector, MLKL, and has been hypothesized to be inflammatory due to its necrotic-like morphology. However, how the immune system "sees" and responds to necroptotic cell death is not well understood. During my dissertation research, we first developed a system to directly activate RIPK3 and trigger necroptosis, independent of upstream signaling. Using this system, we found that RIPK3 activation lead to the upregulation of many pro-inflammatory cytokines and chemokines at the mRNA level, and only a subset of these was made at the protein level. Interestingly, cells that

had undergone necroptosis and were seemingly “dead” were able to continue translating pro-inflammatory mediators. Furthermore, we found that ongoing translation of cytokines and chemokines by “corpses” contributes to necroptotic cell uptake by innate immune cells, as well as priming of adaptive immune responses to antigens associated with necroptotic corpses. The work carried out during my dissertation raises the possibility that necroptosis represents a program in which cell death and inflammatory transcription and translation are coordinated to optimize the immunogenicity of necroptotic cells.

TABLE OF CONTENTS

List of Figures iv

Chapter 1. Introduction 1

1.1 Organization 1

1.1 Programmed Cell Death: An Overview 1

1.2 Pure Death systems 5

1.3 References 7

Chapter 2. RIPK3 in cell death and inflammation 10

2.1 Summary 11

2.2 Introduction 11

2.3 RIPK3-dependent necroptosis: The “canonical” function of RIPK3 12

2.4 RIPK3-dependent apoptosis 16

2.5 The role of RIPK3 in viral infections 17

2.6 RIPK3 and necroptosis in the context of bacterial infection 23

2.7 Contributions of RIPK3 to tissue damage 24

2.8 The role of RIPK3 in inflammation 27

2.9 Concluding remarks 32

2.10 Acknowledgements and funding 33

2.11 References 33

Chapter 3. RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis 47

3.1 Summary 48
3.2 Introduction 48
3.3 Results 51
3.4 Discussion 66
3.5 Materials and methods 69
3.1 Supplementary information 73
3.2 Acknowledgements and funding 79
3.3 References 79

Chapter 4. RIPK3 activation leads to continued cytokine synthesis after loss of cell membrane integrity 85

4.1 Introduction 86
4.2 Results 87
4.3 Discussion 107
4.4 Materials and methods 109
4.5 Supplementary information 116
4.6 Acknowledgments and funding 124
4.7 References 124

Chapter 5. Conclusions 129

5.1 Summary of findings 129
5.2 Ongoing questions 129
5.3 References 131

Appendix A. List of published articles 132

LIST OF FIGURES

Figure 1.1. Morphological changes of apoptosis versus necrosis. 2

Figure 1.2. Signaling downstream of TNFR1 engagement. 5

Figure 1.3. Pure Death system schematic. 6

Figure 1.4. Addition of dimerizer drug (AP1) triggers apoptosis or necroptosis. 7

Figure 2.1. Cell death-dependent and –independent functions of RIPK3. 17

Figure 3.1. RIPK3 dimerization seeds a RHIM-dependent necrosome complex. 54

Figure 3.2. Receptor-independent RIPK3 oligomerization is regulated by RIPK1 and caspase-8. 57

Figure 3.3. Chemically enforced RIPK3 oligomerization activates RIPK3 in the absence of the RHIM domain. 60

Figure 3.4. The presence of RIPK1 inhibits spontaneous RIPK3 oligomerization and necroptosis. 64

Figure 3.5. Catalytically inactive RIPK1 mimics the suppressive effects of Nec-1 on spontaneous RIPK3 oligomerization and necroptosis. 66

Figure 3.6. Orozco *et al.* CDD 2014, Supplementary Figure 1. 75

Figure 3.7. Orozco *et al.* CDD 2014, Supplementary Figure 2. 77

Figure 3.8. Orozco *et al.* CDD 2014, Supplementary Figure 3. 79

Figure 4.1. RIPK3 activation leads to ongoing translation even after cell membrane permeabilization. 92

Figure 4.2. Zombie cytokine translation occurs within corpses. 94

Figure 4.3. Zombie translation is associated with the ER in necroptotic corpses. 97

Figure 4.4. Infection with MCMV M45ΔRHIM leads to necroptosis and zombie translation. 100

Figure 4.5. Necroptotic cells that have lost membrane integrity preferentially translate cytokine and chemokine mRNA. 102

Figure 4.6. Zombie translation influences myeloid cell trafficking and cross-priming potential necroptotic cell-derived antigen. 107

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Andrew Oberst, for his support and guidance over the years. Andrew has been very generous in encouraging me to go to multiple international conferences, in establishing fruitful collaborations, and allowing me to engage in networking opportunities. More importantly, Andrew has provided an endless amount of encouragement and inspiration, even when I felt everything was falling apart. "It's a game-changer, Orozco!" As one of his first graduate students, I had the pleasure of seeing him grow as a mentor, and I am very excited for what the Oberst Lab will accomplish moving forward.

I also want to thank my committee members: Drs. Julie Overbaugh, Keith Elkon, Edward Clark, and Adam Lacy-Hulbert. My committee members not only supported and encouraged me, but gently course-corrected and guided me. I am definitely a better, more creative scientist thanks to them.

Thank you to members of the Oberst Lab, past and present. Margo Werner was indispensable in keeping me sane my first couple of years in graduate school, and she inspired me to be a badass. Tayla Olsen and Pooja Jain have been wonderful lab managers over the years, helping me complete experiments and providing me with coffee and comfort when things weren't going right. Dr. Brian Daniels was exceptional in providing mentorship, sass, and a good schooling on statistics; Dr. Michelle Messmer provided kindness, patience, and knowledge on dendritic cells and beyond; Annelise Synder taught me hilarity, resilience, what it means to be organized, and how to design a good flow panel. And last but not least, thank you to Dr. Michelle Brault, my twin, my partner in crime, my science buddy. Grad school wouldn't have been the same without her.

A huge thank you goes to the staff of both the Molecular and Cellular Biology graduate program, and the Immunology graduate program – for keeping me accountable, answering dumb questions, and guiding me through this process called graduate school.

I am so grateful to my other mentors: my scientific soulmate, Dr. Nader Yatim, for teaching and guiding me for three wonderful weeks at the Pasteur Institute, and letting me bother him via email every other week. I learned so much from him, and am inspired to do better work because of him. Thank you to Dr. Matthew Albert for not only being a great mentor, but for welcoming me into his lab and allowing me to work with Nader and engage with a number of amazing scientists. Thank you to Drs. Robert Beatty and Eva Harris, for believing in me and encouraging me to go to graduate school. I will forever consider you both my mentors, and be indebted for giving a young woman with no research experience a chance.

Finally, I would like to thank my friends and family. My friends Meara and Edd Wilder-James have provided immense encouragement and lovely visits full of food, music, hilarity, and rest. My family in Mexico has provided love and encouragement, and lots of questions from afar. My parents, Sharon Steeber and Salvador Orozco, have always been extremely supportive and believed that I could do anything I put my mind to. I want to thank my brother Christopher for

helping me keep things in perspective. Thank you to my grandmother Alice Steeber for being my angel, and to Donna and Joe Hynes for keeping me sane and lighthearted, and well supplied with wine. It is because of all of you that I made it here.

DEDICATION

To my mother, Sharon Steeber, who taught me the importance of hard work, creativity, kindness, and the futility of trying to keep a desk free of paper piles.

Chapter 1. INTRODUCTION

1.1 ORGANIZATION

The original aim of this work was to develop a system by which to induce programmed cell death and analyze the subsequent immunological outcomes. In chapter 1, I give a brief introduction to apoptosis and necroptosis, two distinct forms of programmed cell death. In chapter 2, I review the pro-necroptotic protein, RIPK3, and its roles in both cell death and inflammation. In chapter 3, I introduce our activatable RIPK3 system, and describe some interesting results we found using this system. Chapter 4 consists of a number of findings, both published and unpublished, that demonstrate that different forms of cell death are not created immunologically equal. In chapter 5, I describe how necroptotic corpses are capable of "zombie translation," and how this influences and shapes subsequent immune responses. Finally, in chapter 6, I summarize the findings in this dissertation and discuss unanswered questions and potential clinical applications.

1.1 PROGRAMMED CELL DEATH: AN OVERVIEW

Historically, cell death has been characterized as either apoptosis, a form of programmed cell death (PCD) dependent on caspases, or necrosis, a form of passive cell death caused by overwhelming chemical or environmental insult. These two forms of cell death differ morphologically; apoptosis is characterized by nuclear fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing into apoptotic bodies (*1*). During necrosis, on the other hand, the cell swells and bursts, leading to a release of intracellular contents (Fig. 1.1).

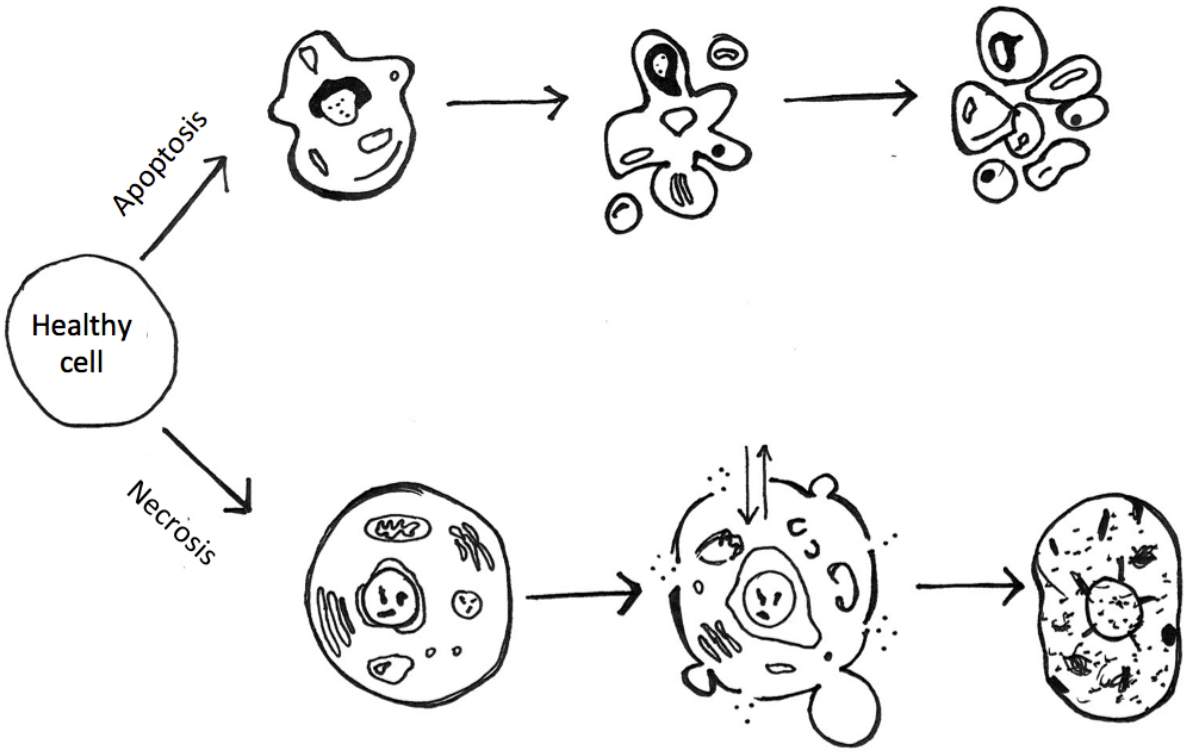


Figure 1.1. Morphological changes of apoptosis versus necrosis.

Apoptosis is dependent on a family of proteases termed caspases (*2*). There are two main apoptotic signaling pathways: the extrinsic and the intrinsic pathway (*1*). For the purposes of this dissertation, I will focus on the extrinsic pathway of apoptosis, although the pathways ultimately converge on the same executioner proteins. In the extrinsic pathway, apoptosis is initiated by death receptor (DR) ligation; members of this superfamily include the tumor necrosis factor receptor 1 (TNFR1) and Fas receptor (FasR). DR engagement leads to the activation of procaspase-8, an initiator caspase that can cleave and activate executioner caspases. Caspase activation results in the cleavage of hundreds of cellular substrates and ultimately leads to cell demise. During apoptotic cell death, there is mRNA decay, as well as inhibition and shutdown of protein translation (*3, 4*).

In the last decade, another form of PCD termed "necroptosis" has been described. Unlike apoptosis, necroptosis is a caspase-independent process, and is instead reliant on the serine/threonine kinase receptor-interacting protein kinase-3 (RIPK3)(*5-7*) and its downstream effector, mixed lineage kinase domain-like (MLKL)(*8-11*). Necroptosis shares morphological similarities with necrosis; upon execution of the necroptotic program, cells swell due to disrupted ion homeostasis and ultimately explode.

Apoptosis and necroptosis can be induced by numerous physiological stimuli, such as through stimulation of DRs, and in fact, their signaling pathways are often intertwined (described in Chapter 2). For the sake of simplicity and brevity, I will describe signaling downstream of one such DR, TNFR1. Tumor necrosis factor alpha (TNFα) is the ligand for TNFR1, and while this stimulus is generally considered pro-inflammatory, it can have a number of different outcomes. Upon TNFR1 engagement, an early complex forms that includes RIPK1, the cellular inhibitors of apoptosis (cIAPs), and the linear ubiquitin chain assembly complex (LUBAC), leading to the activation of NF-κB; this typically results in cell survival, proliferation, cytokine and chemokine production, as well as inflammation (*12*).

However, a second, cytosolic complex including FAS-associated death domain protein (FADD), RIPK1, and the pro-apoptotic caspase-8 can form, thus leading to caspase-8 activation and cleavage of the downstream targets caspase-3 and caspase-7 (which are executioner caspases) – therefore, the cell will succumb to apoptotic death. So why do cells usually survive TNFα treatment? Upon NF-κB activation, one of the pro-survival molecules that is rapidly upregulated is the caspase-8 homolog, cFLIP (*13*), which can bind to and alter caspase-8 activity, leading to cell survival; in fact, treatment of cells *in vitro* with TNFα and either a transcription or translation inhibitor will lead to apoptosis, due to the cell's inability to upregulate cFLIP (*14*).

In the absence or inhibition of caspase-8, RIPK1 can go on to form a complex with RIPK3 via their shared RIP homotypic interaction motif (RHIM). Within this complex, termed the "necrosome"(*15*), RIPK3 becomes active via phosphorylation and ultimately recruits and phosphorylates the executioner MLKL. Active MLKL oligomerizes and is thought to localize to the plasma membrane, where it disrupts plasma membrane integrity through unclear mechanisms. When studied *in vitro,* the pan-caspase inhibitor zVAD.fmk is used in combination with TNFα treatment to trigger necroptosis; again, this is because it targets the catalytic of caspase-8. In fact, the catalytic activity of the caspase-8 and cFLIP complex not only blocks apoptosis, but necroptosis as well (*16*). Thus, TNFα stimulation is complex and can lead to different outcomes, depending on the cellular context (Fig. 1.2).

Although the ultimate outcome of either apoptosis or necroptosis is cellular demise, *how* a cell dies seems to determine how the immune system perceives and reacts to the cell death event. Apoptosis is generally considered immunogenically silent or even tolerogenic, and apoptotic bodies are difficult to detect in healthy tissue as they are rapidly cleared by phagocytes (*17, 18*). In contrast, researchers in the cell death field have commonly hypothesized that necroptosis is inflammatory due to its morphological features – the cell swells, bursts, and releases its intracellular contents. These released molecules, which are termed DAMPs (damage- or danger-associated molecular patterns), can be sensed by neighboring phagocytes and innate immune cells to elicit an immune response (*19*). Examples of DAMPs include the chromatin-associated protein HMGB1, extracellular ATP, and DNA (*20*). Despite the overarching hypothesis that necroptosis is inflammatory, the details of how apoptotic and necroptotic cells are perceived by different immune cells are unclear.

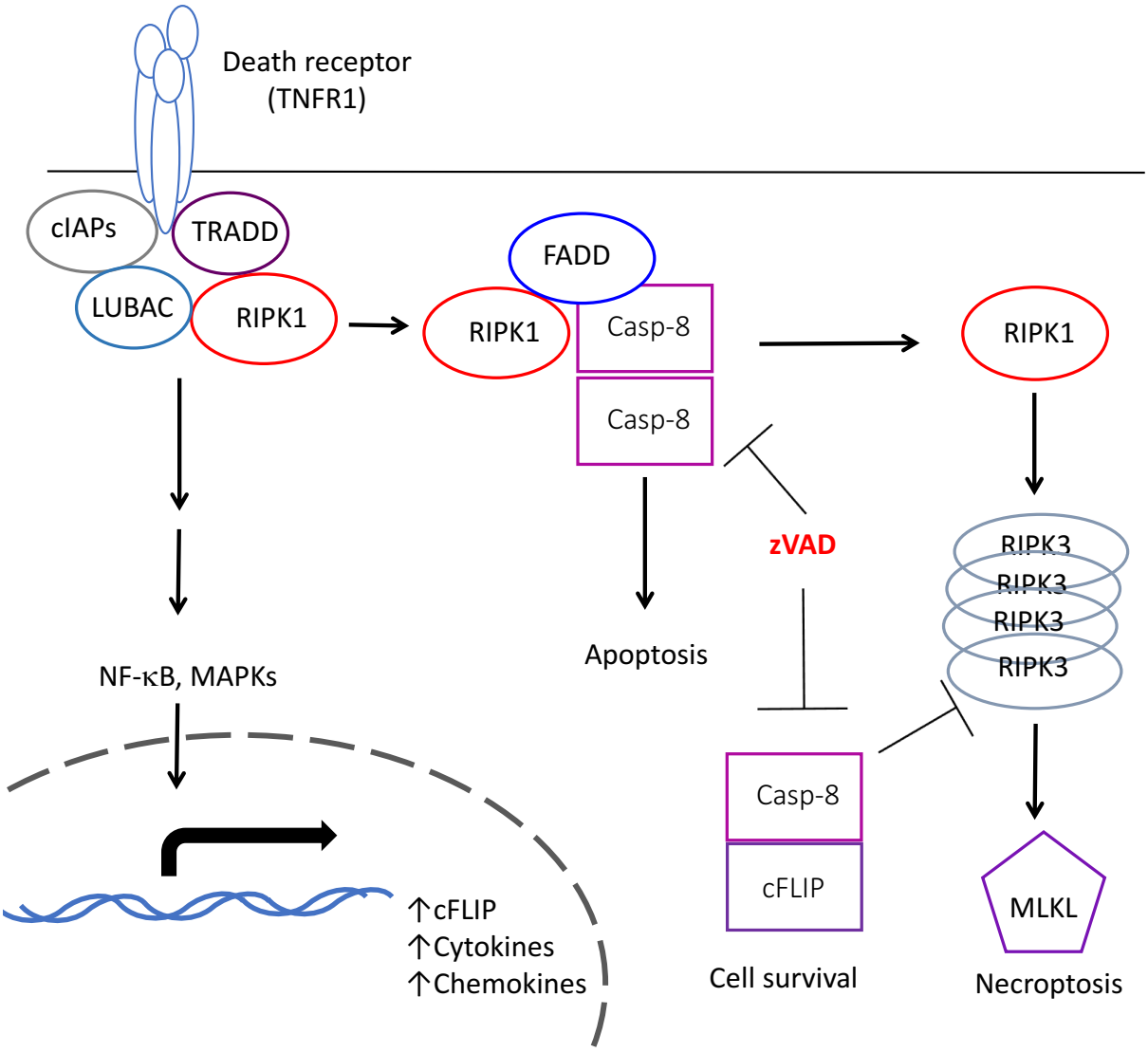


Figure 1.2. Signaling downstream of TNFR1 engagement.

1.2 Pure Death Systems

Given the complicated nature of the signaling pathways that lead to cell survival, apoptosis, or necroptosis, we sought to develop a system by which to directly activate the effector molecules of different cell death programs, thus inducing a "pure" death program. To do this, we added a version of the FKBP protein (mutated at F36V, here named "FV" domain) to

caspase-8, or added two tandem C-terminal FV domains to RIPK3 with or without the RHIM domain (RIPK3-2xFV or RIPK3ΔC-2xFV, respectively) (Fig 1.3).

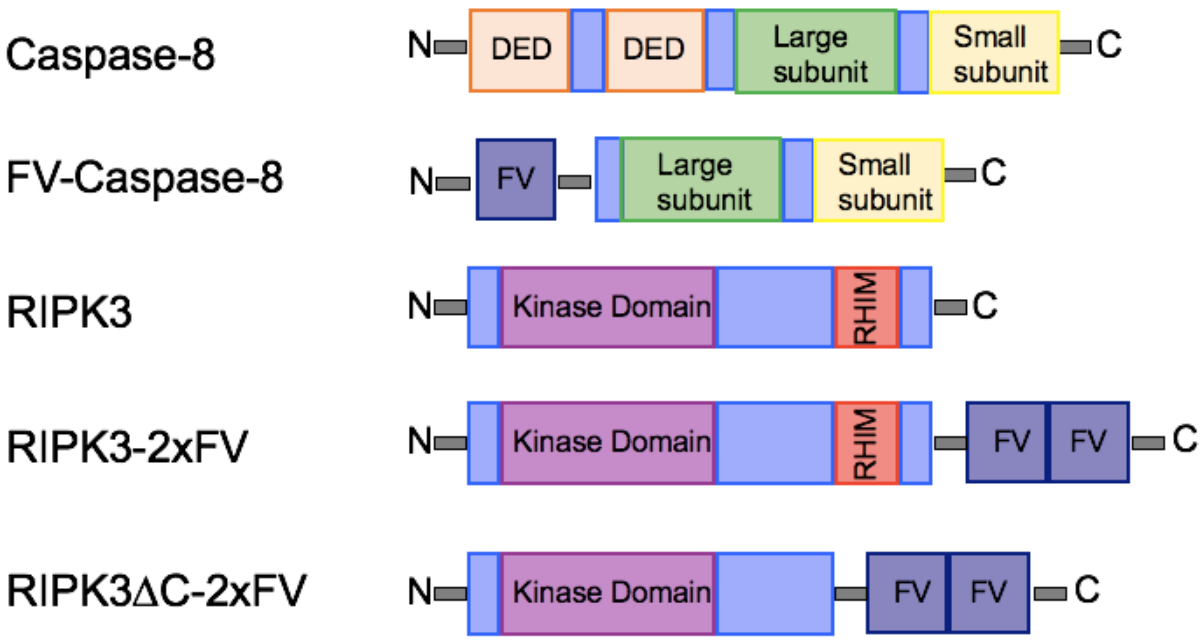


Figure 1.3. Pure Death system schematic.

We expressed these constructs in NIH-3T3 fibroblasts, and upon addition of a small nontoxic drug (dimerizer drug, or AP1), we were able to trigger specific forms of programmed cell death (described in more detail in chapter 4) (Fig. 1.4). Our goal was to use this reductionist approach to study the immunological consequences to both apoptosis and necroptosis, without engaging the complicating upstream signaling. I used these systems throughtout my dissertation research to investigate a number of questions.

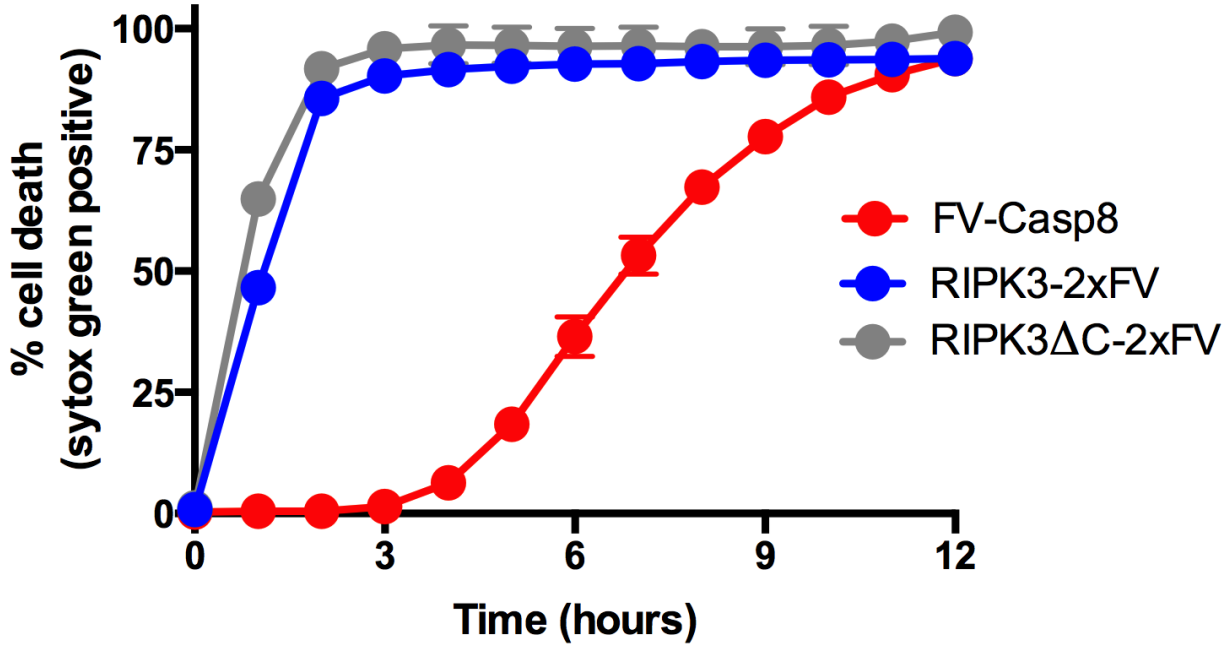


Figure 1.4. Addition of dimerizer drug (AP1) triggers apoptosis or necroptosis.

1.3 REFERENCES

1. S. Elmore, Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35**, 495-516 (2007).

2. N. A. Thornberry, Caspases: key mediators of apoptosis. *Chem Biol* **5**, R97-103 (1998).

3. M. Bushell, W. Wood, M. J. Clemens, S. J. Morley, Changes in integrity and association of eukaryotic protein synthesis initiation factors during apoptosis. *Eur J Biochem* **267**, 1083-1091 (2000).

4. M. Bushell, M. Stoneley, P. Sarnow, A. E. Willis, Translation inhibition during the induction of apoptosis: RNA or protein degradation? *Biochem Soc Trans* **32**, 606-610 (2004).

5. Y. S. Cho *et al.*, Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* **137**, 1112-1123 (2009).

6. S. He *et al.*, Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* **137**, 1100-1111 (2009).

7. D. W. Zhang *et al.*, RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* **325**, 332-336 (2009).

8. L. Sun *et al.*, Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* **148**, 213-227 (2012).

9. J. Zhao *et al.*, Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 5322-5327 (2012).

10. W. Chen *et al.*, Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling. *The Journal of biological chemistry* **288**, 16247-16261 (2013).

11. J. Wu *et al.*, Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. *Cell Res* **23**, 994-1006 (2013).

12. A. T. Ting, M. J. M. Bertrand, More to Life than NF-kappaB in TNFR1 Signaling. *Trends Immunol* **37**, 535-545 (2016).

13. O. Micheau, S. Lens, O. Gaide, K. Alevizopoulos, J. Tschopp, NF-kappaB signals induce the expression of c-FLIP. *Mol Cell Biol* **21**, 5299-5305 (2001).

14. S. Kreuz, D. Siegmund, P. Scheurich, H. Wajant, NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. *Mol Cell Biol* **21**, 3964-3973 (2001).

15. J. Li *et al.*, The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. *Cell* **150**, 339-350 (2012).

16. A. Oberst *et al.*, Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* **471**, 363-367 (2011).

17. K. L. Rock, H. Kono, The inflammatory response to cell death. *Annu Rev Pathol* **3**, 99-126 (2008).

18. N. Yatim, S. Cullen, M. L. Albert, Dying cells actively regulate adaptive immune responses. *Nature reviews. Immunology* **17**, 262-275 (2017).

19. H. Kono, K. L. Rock, How dying cells alert the immune system to danger. *Nature reviews. Immunology* **8**, 279-289 (2008).

20. L. Schaefer, Complexity of danger: the diverse nature of damage-associated molecular patterns. *The Journal of biological chemistry* **289**, 35237-35245 (2014).

Chapter 2. RIPK3 IN CELL DEATH AND INFLAMMATION

This chapter is based on the following publication:

Orozco S, Oberst A. RIPK3 in cell death and inflammation: the good, the bad, and the ugly. *Immunol Rev.* 2017;277:102-112. https://www.ncbi.nlm.nih.gov/pubmed/28462521

2.1 SUMMARY

Necroptosis is a form of cell death that can be observed downstream of death receptor or pattern recognition receptor signaling under certain cellular contexts, or in response to some viral and bacterial infections. The receptor interacting protein kinases-1 (RIPK1) and RIPK3 are at the core of necroptotic signaling, among other proteins. Because this pathway is normally halted by the pro-apoptotic protease caspase-8 and the IAP ubiquitin ligases, how and when necroptosis is triggered in physiological settings are ongoing questions. Interestingly, accumulating evidence suggests that RIPK3 has functions beyond the induction of necroptotic cell death, especially in the areas of tissue injury and sterile inflammation. Here, we will discuss the role of RIPK3 in a variety of physiological conditions, including necroptotic and non-necroptotic cell death, in the context of viral and bacterial infections, tissue damage, and inflammation.

2.2 INTRODUCTION

Billions of cells die in multicellular organisms each day as part of tissue homeostasis and clearance of old or damaged cells. The majority of these cell death events occur via apoptosis, a well-described form of programmed cell death dependent on the caspases, a family of cysteine-aspartic proteases (1). Apoptosis is generally considered immunologically silent or even tolerogenic; the immune system typically does not perceive this form of cell death as a threat, and cells that have undergone apoptosis are quickly and neatly cleared by local phagocytes. Historically, apoptosis has been contrasted with the unprogrammed process of necrosis, in which cells are killed by overwhelming chemical or environmental insult. Necrosis involves cell lysis and the release of reactive molecules normally contained by the plasma membrane, including a nebulous class of molecules called danger (or sometimes “damage”)-associated molecular patters

(DAMPs). DAMPs are perceived as signatures of damage by the innate immune system, and can trigger inflammatory responses; for this reason, necrosis is considered to be a driver of inflammation (2).

In recent years, the dichotomy between programmed, non-inflammatory apoptosis and unprogrammed, inflammatory necrosis has been challenged by the description of additional forms of cell death that are both programmed and inflammatory. Among these is the process of "programmed necrosis," also called necroptosis. Necroptosis shares morphological similarities with passive necrosis: cells undergoing necroptosis swell and burst, leading to the release of DAMPs (3, 4). This morphological similarity has led to the idea that necroptosis, like necrosis, is an inflammatory form of cell death. Necroptosis is triggered by activation of the RIP kinases, RIPK1 (5, 6) and RIPK3 (7-9). Importantly, recent work has indicated that activation of these kinases can trigger transcriptional responses in addition to—and in some cases accompanying—necroptotic cell death. Furthermore, evidence from knockout mice implies that the lytic nature of necroptosis may not be the key driver of inflammation and immune responses to cell death, and that inflammatory transcriptional signaling carried out by the RIP kinases in dying cells may determine how necroptotic cells are perceived by the immune system. In this review, we will discuss the inflammatory effects of necroptotic cell death, and evidence for the relative contributions of DAMPs and transcriptional responses to necroptotic stimuli. We will highlight the signaling underlying this cell death program, as well as non-death outcomes of RIPK activation.

2.3 RIPK3-DEPENDENT NECROPTOSIS: THE "CANONICAL" FUNCTION OF RIPK3

The pathway by which cells are induced to die by necroptosis has been worked out in detail in recent years, and has been extensively described elsewhere (10-12). Here we present an

abbreviated description of this pathway, in an effort to introduce the key enzymes and regulatory mechanisms involved. The core of the necroptotic pathway involves the activities of the RIP kinases, RIPK1 and RIPK3, and the phosphorylation-driven activation by the latter of the pseudokinase mixed lineage kinase domain-like (MLKL).

RIPK1 and RIPK3 interact with each other via the RIP homotypic interaction motif (RHIM) that both possess. RHIM-RHIM interactions between RIPK1 and RIPK3 lead to formation of the "necrosome," an oligomeric cytosolic complex in which reciprocal phosphorylation between RIPK1 and RIPK3 (13) can lead to MLKL recruitment and activation. Importantly, in addition to a RHIM domain, RIPK1 also contains a death domain (DD) through which it can recruit the adapter FADD, the protease caspase-8, and the caspase paralog cFLIP to the necrosome. Recruitment of these proteins by RIPK1, along with the activity of the IAP ubiquitin ligases, inhibits RIPK1/RIPK3 oligomerization and signaling and prevents necroptosis (14-17). For this reason, necroptosis is generally observed in conditions in which caspase-8 and/or the IAPs are absent or inhibited. Consistent with this, genetic ablation of caspase-8 leads to embryonic lethality in mice due to hyperactivation of necroptosis during development; this lethality is rescued by co-ablation of RIPK3 (16, 18).

Upon necrosome-driven activation, RIPK3 phosphorylates the pseudokinase MLKL, which serves as the executioner of necroptosis (19-23). Phosphorylation of MLKL leads to a conformational switch that exposes its N-terminal four-helix bundle domain and leads to the oligomerization of MLKL (23-27). It has been proposed that this oligomerization leads to a net positive charge of the multi-MLKL complex, and therefore active MLKL is recruited to negatively charged membranes, leading to membrane disruption (28). While the exact mechanism by which MLKL leads to cell membrane disruption is still unclear, it is well

appreciated that MLKL is essential for necroptotic death to occur (27). Notably, knockout of MLKL also rescues the embryonic lethality observed in caspase-8 knockout animals, consistent with the idea that caspase-8 deficiency causes embryonic lethality via RIPK3- and MLKL-mediated necroptosis (29).

If the necrosome represents the core of the necroptotic pathway, what signals control its formation? Necrosome formation involves RHIM-dependent interactions, and the activation of RHIM-containing proteins is therefore required to initiate necroptotic signaling. Four RHIM-containing proteins have been described in the mammalian proteome: in addition to RIPK1 and RIPK3, the adaptor protein TRIF contains a RHIM, and the innate immune sensor DNA-dependent activator of IFN-regulatory factors (DAI) contains three putative RHIMs (30). RIPK3 acts as a signaling adaptor for the activation of MLKL (among other functions, discussed below), and signals leading to RIPK3 activation involve activation of RIPK1, TRIF, and/or DAI. We'll briefly consider each of these signaling pathways here, and will discuss specific physiological settings in which they can be activated later in this review.

RIPK1 participates in a wide array of receptor signaling pathways, notably those initiated by the "death receptors" TNFR1, Fas, and the TRAIL receptors (31, 32). In these contexts, RIPK1 has a kinase-independent role in the initiation of inflammatory transcriptional programs mediated by NF-κB (33, 34) and the MAP kinases (35, 36). However, these receptor signals can also lead to translocation of RIPK1 into the cytosol, where it can interact with necrosome components. As discussed above, whether cytosolic RIPK1 is able to form a stable complex with RIPK3 to trigger necroptosis depends on the status of caspase-8 and cFLIP, which inhibit this process. Importantly, upon receptor activation, cFLIP is upregulated by the inflammatory transcription programs in which RIPK1 participates (37, 38); this represents one mechanism by

which the pro-inflammatory activity of RIPK1 exerts feedback inhibition on its pro-death activity. This also highlights a theme to which we will return repeatedly: that the signals that initiate necroptosis can also drive inflammatory transcription.

Necroptosis can also be triggered by TRIF (39, 40), a signaling adaptor that contains both a TIR domain and a RHIM. TRIF is activated downstream of TLR3 and TLR4, which sense double-stranded RNA (dsRNA) or lipopolysaccharide (LPS), respectively. Analogously to TNFR1, signaling through either of these TLRs normally leads to pro-inflammatory and pro-survival responses in the cell due to NF-κB and IRF-3 activation (41), but when cells are sensitized to necroptosis by inhibition of capase-8/cFLIP, TRIF can activate RIPK1 and/or RIPK3. Notably, because TRIF contains a RHIM domain, it can directly activate RIPK3 in the absence of RIPK1 (39, 40); however, RIPK1 can also be recruited to TRIF (42), and this recruitment may alter the outcome of TRIF-RIPK3 signaling by promoting transcriptional responses and engaging the inhibitory effects of caspase-8 and cFLIP (recall that the latter depend on the DD of RIPK1 for recruitment). Again, necroptotic signaling is initiated by proteins— TRIF and RIPK1— that can also drive inflammatory transcription.

The final RHIM containing protein encoded by mammals is DAI (also known as ZBP1 or DLM-1). DAI was initially described as a sensor of DNA in the unusual Z-form conformation (Z-DNA) (43), and has since been shown to elicit cell death and transcriptional responses to DNA viruses via its Z-DNA binding domain (44, 45). However, more recent data have shown that DAI can trigger necroptosis downstream of influenza A virus (IAV)(46, 47), a single-stranded negative-sense segmented RNA virus (discussed later in this review), as well as in sterile settings in which the ability of RIPK1's scaffolding function to suppress RIPK3 is

disrupted (48, 49). As neither of these functions is consistent with the sensing of Z-DNA, it remains unclear how DAI is activated in these settings.

2.4 RIPK3-DEPENDENT APOPTOSIS

In addition to the induction of necroptosis, the necrosome can also trigger apoptosis in some settings; this effect depends on the recruitment of caspase-8, and appears to predominate when RIPK3 is inactive or MLKL is absent, precluding necroptotic signaling. In this context, RIPK3 serves as a pro-apoptotic adaptor and recruits RIPK1 and FADD, forming a platform that leads to the activation of caspase-8 and subsequently, apoptosis (50, 51). In fact, while RIPK3-deficient animals develop normally (52), mice engineered to express a version of catalytically inactive RIPK3 (D161N) die at day E11.5 from aberrant apoptosis. *Casp8*$^{-/-}$*Ripk3*$^{D161N/D161N}$ mice are viable, demonstrating the role of caspase-8 in promoting apoptosis during embryogenesis of these animals (50). Similar effects are observed when a small-molecule inhibitor of RIPK3 is used (51). Of interest, mice with a different knock-in version of catalytically inactive RIPK3 (K51A) not only survive to birth, but are viable, fertile, and immunocompetent, and phenocopy *Ripk3*$^{-/-}$ mice in rescuing the embryonic lethality of *Casp8*$^{-/-}$ animals (51). Importantly, while RIPK3^{K51A} does not induce RIPK3-dependent apoptosis in the manner observed with RIPK3^{D161N}, treatment of cells expressing RIPK3^{K51A} with RIPK3 inhibitors unleashes a pro-apoptotic activity even in the K51A mutant (51). Together, these findings lead to a model in which chemical inhibition of RIPK3, or presence of the D161N mutation, alters the conformation of RIPK3 stabilizes the necrosome but prevents signaling to MLKL. This platform is thereby able to mediate sustained interactions with RIPK1 and caspase-8, promoting apoptosis (Figure 1). Interestingly, a similar form of RIPK3-dependent "reverse signaling" was recently described during influenza infection, as described below.

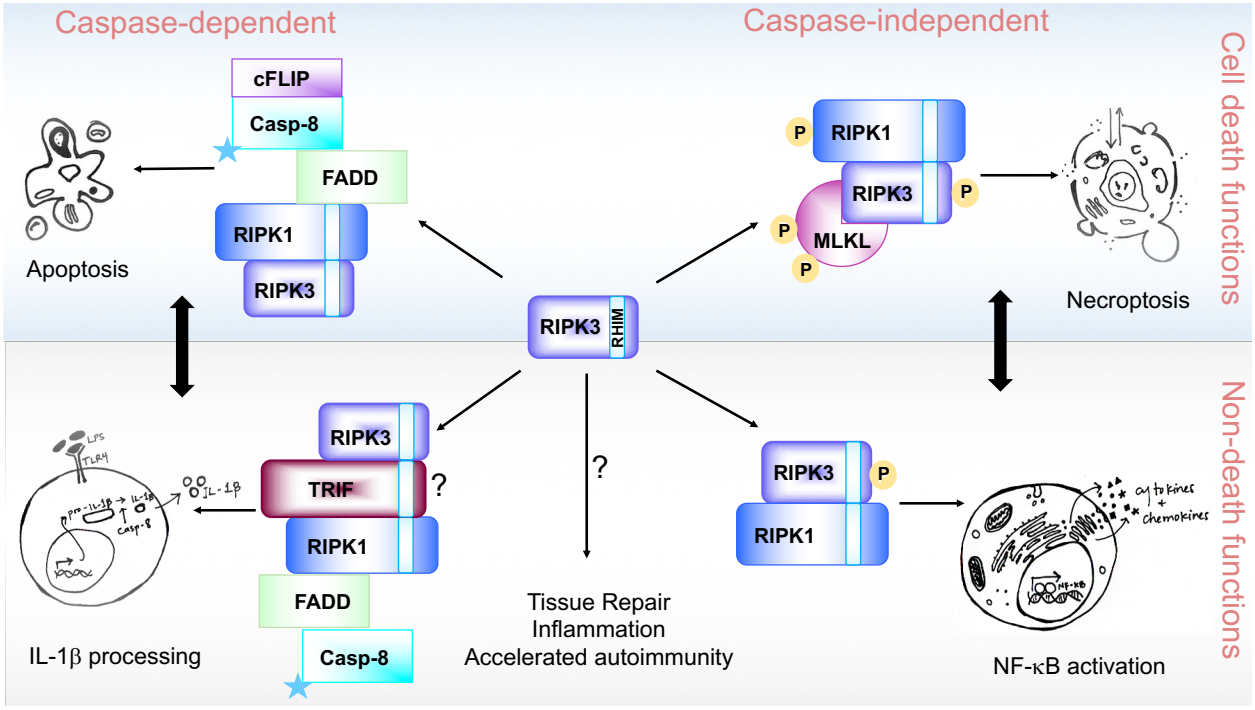


Figure 2.1. Cell death-dependent and –independent functions of RIPK3.
RIPK3 has been implicated in the induction of both apoptosis and necroptosis, as well as cell death-independent roles in inflammatory transcription and IL-1β processing. These different functions have been associated with distinct protein complexes, though importantly it remains unclear to what degree the components of these complexes are actually distinct. This figure depicts the multiple functions ascribed to RIPK3, and the relevant protein interactors. These include induction of cell death (top blue panel) and/or inflammatory outcomes (bottom gray panel). RIPK3-dependent processes can be further divided into caspase-dependent and caspase-independent contexts, here shown on the left and right of the figure respectively. It is important to note that the cell death and non-death functions of RIPK3 are not necessarily mutually exclusive, and in fact, cell death and inflammation may be a coordinated response (dark black arrows). For example, NF-κB activation has been shown to accompany necroptotic cell death in a model of direct RIPK3 activation, and the resulting transcriptional program is necessary for the immunogenicity of antigen derived from necroptotic cells (93). Similarly, IL-1β processing is often accompanied by caspase-dependent cell death. Stars represent catalytic activity of caspase-8.

2.5 THE ROLE OF RIPK3 IN VIRAL INFECTIONS

Having defined the core pathways leading to necrosome formation, the question arises: when are these pathways engaged physiologically? As noted, necrosome formation and apoptosis are antagonized by the caspase-8/FLIP complex and the IAPs in many settings, so when does

necroptosis occur under natural conditions? Mice lacking RIPK3 are particularly susceptible to certain viruses, providing genetic evidence that RIPK3 is important in controlling viral infections (7, 44, 53). However, there have been hints that RIPK3 has functions beyond cell death, and that its activation can lead to transcriptional responses (discussed later), which may also contribute to the clearance of viruses. Thus, in some situations it is unclear whether RIPK3 is important in viral clearance because of cell death, transcriptional functions, or a combination of both. Ongoing efforts to compare *Ripk3*$^{-/-}$ animals to mice lacking MLKL and/or caspase-8 will help to clarify this point.

One of the first studies to demonstrate that RIPK3 is important in the control of viral infection was also one of the first to pinpoint RIPK3 as a key protein in the necroptotic pathway. In 2009, Cho and colleagues used an RNA interference screen to identify RIPK3 as a pro-necrotic kinase activated during vaccinia virus (VacV) infection (7). This seminal study found that wild-type (WT) T cells infected with VacV were sensitive to TNF-induced necroptosis, while RIPK3-deficient T cells were protected. Furthermore, they demonstrated that RIPK3-deficient mice had higher viral tissue titers upon VacV infection compared to their WT counterparts, illustrating the role of RIPK3 in controlling VacV replication. Interestingly, a much earlier study found that VacV infection of murine fibroblasts sensitized these cells to a "necrotic-like" death upon TNF treatment, and that this sensitization required expression of the viral caspase inhibitor, B13R (54). This finding predated the clear description and naming of necroptosis, but it seems clear that the authors were observing necroptotic cell death. Together, these findings provide compelling evidence that RIPK3-dependent necroptosis can occur when caspases, and therefore apoptosis, are blocked by viral inhibitors, and that this cell death pathway contributes to the control of VacV infection.

The idea that necroptosis provides protection against infection by DNA viruses is reinforced by the finding that murine cytomegalovirus (MCMV) encodes a RHIM-containing inhibitor of necroptosis, as well as an inhibitor of caspase activation. The MCMV M36 gene encodes a caspase-8 inhibitor (viral inhibitor of caspase-8-induced apoptosis, or vICA)(55); while blockade of caspase-8 would normally prime a cell for necroptosis, the MCMV M45 gene encodes an inhibitor of RIP activation (vIRA)(56). vIRA contains a RHIM domain, and as such, can inhibit RIPK3-dependent necrosis (57). In fact, only MCMV strains that lack vIRA, or that express a RHIM-mutant version of vIRA (M45*mut*RHIM) can induce necroptosis, as vIRA is no longer able to block RIPK3 activation. These viruses are attenuated in mice (58, 59); however, this attenuation is reversed in RIPK3-deficient mice (58), demonstrating that necroptosis can control viral infection, and that vIRA is essential for MCMV to block RIPK3-dependent necrosis. Importantly, in 2012 it was demonstrated that DAI, a PRR thought to sense the DNA of viral genomes, sensitizes cells to necroptosis upon MCMV infection (44). DAI contains three RHIM domains, and RHIM-A is responsible for mediating interactions between DAI and RIPK3. Upton *et al.* demonstrated that DAI and RIPK3 form a complex during MCMV infection and this leads to necroptosis; in fact, like in RIPK3-deficient mice, MCMV M45*mut*RHIM virus attenuation is also reversed in mice lacking DAI (44).

Like its murine counterpart, human CMV (HCMV) encodes inhibitors of both apoptosis and necroptosis. HCMV *UL36* encodes vICA, which is important for the suppression of apoptosis in infected human cells (60). Curiously, however, an HCMV immediate early 1 (IE1) gene product is responsible for suppressing necroptosis downstream of RIPK3 phosphorylation and activation of MLKL, although the specific identity of the inhibitor is unknown (61). This differs from MCMV's strategy for blocking necroptosis, which utilizes vIRA to inhibit RIPK3

activation through a RHIM-dependent mechanism. This finding is interesting for at least two reasons: first, it highlights the diversity of strategies employed by viruses to inhibit necroptosis. Second, the finding that the inhibition mediated by HCMV targets MLKL activation rather than necrosome formation identifies RIPK3- and MLKL-dependent cell death, rather than other potential aspects of necrosome signaling, as the target of viral inhibition in this setting.

Herpes simplex virus (HSV)-1 and HSV-2 are other examples of human pathogens that have evolved to inhibit cell death pathways during infection (62, 63). The *UL39* gene of HSV-1 encodes the protein ICP6, which is also known as HSV-1 ribonucleotide reductase (RNR) subunit 1 (R1). Likewise, HSV-2 encodes a similar protein, ICP10. ICP6 and ICP10 are interesting because they cannot only block apoptosis through their C-terminal caspase-8-binding domain, but can also block necroptosis by inhibiting RIPK1 and RIPK3 interactions via their own N-terminal RHIM domain (64, 65). Although this mechanism of necroptosis suppression is reminiscent of vIRA encoded by MCMV, it is interesting to note that in the case of HSV, a single gene product can impede both apoptosis and necroptosis in human cells. Notably, both ICP6 and ICP10 have been shown to activate, rather than inhibit, necroptosis in murine cells through a RHIM-dependent mechanism, demonstrating a species specificity of RHIM signaling outcome (65).

Although the examples noted thus far have pointed to a role for DAI- and RIPK3-mediated necroptosis in eliminating DNA viruses, RIPK3-dependent cell death has recently been shown to be important for the control of the segmented RNA virus influenza type A (IAV). In addition to showing that cells lacking RIPK3 are resistant to IAV-induced death, Nogusa *et al.* demonstrated that IAV infection leads to both apoptotic and necroptotic signaling from the necrosome, in a manner analogous to the “reverse signaling” observed upon RIPK3 inhibition

(discussed above)(53). Upon IAV infection, mouse embryonic fibroblasts (MEF) were found to undergo necroptosis; however, in the absence of MLKL, IAV infection instead triggered apoptosis, which depended on RIPK3, RIPK1, FADD and caspase-8. Interestingly, the authors found that necroptosis contributed to IAV control, but that RIPK3-dependent apoptosis could compensate for loss of necroptosis, as illustrated by the fact that both RIPK3-deficient mice and *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ double knockout (DKO) mice (which are deficient in both apoptotic and necroptotic signaling), are more susceptible to IAV than either WT or *Mlkl*$^{-/-}$ mice alone (53). It is interesting that in the case of IAV, MLKL-deficiency does not phenocopy RIPK3-deficiency in mice, and that RIPK3-dependent apoptosis contributes to protection against this virus when MLKL is absent. Given that CMV and HSV encode inhibitors of apoptotic and necroptotic signaling, future studies using viruses lacking both inhibitors may clarify the relative contribution of apoptotic and necroptotic necrosome signaling to viral control *in vivo.*

Subsequently, multiple groups demonstrated that IAV activates RIPK3 and triggers necroptosis through DAI—a molecule that that was previously thought to directly sense DNA (46, 47). How DAI senses IAV infection is somewhat disputed, however; one initial report indicated that DAI senses the viral proteins NP and PB1 (46), while another indicated that DAI recognizes and binds nascent IAV RNA genomes (47). The contribution of DAI— and indeed of necroptosis itself—in controlling IAV infection *in vivo* is also somewhat unclear. DAI-deficient mice have been shown to be either susceptible or resistant to IAV infection by different groups (46, 47), and while Nogusa et al. found that RIPK3-deficient mice exhibited increased susceptibility to IAV (53), an earlier report also showed that excess RIPK3 activation could cause detrimental tissue damage during IAV infection in mice lacking cIAP2 in the lung (66).

As noted previously, RIPK3 may have functions beyond cell death in the context of viral infections. Interestingly, Harris and colleagues demonstrated a temporal role of RIPK3 in intestinal epithelial cells (IECs) infected with the RNA virus coxsackievirus B3 (CVB)(67). Early in infection, RIPK3 facilitates CVB replication by regulating flux through the autophagic pathway, necessary for the life cycle of the virus. At later points of infection, however, the CVB-encoded cysteine protease $3C^{pro}$ cleaves RIPK3, rendering it incapable of transducing the necroptotic signal. Of interest, the C-terminal RHIM-containing cleavage product of RIPK3 was shown to induce non-necrotic cell death, while the N-terminal kinase domain-containing product was unable to trigger any form of cell death (67). Although it is unclear what type of non-necrotic cell death occurs once $3C^{pro}$ cleaves RIPK3, it is tempting to speculate that perhaps the fragment of RIPK3 containing the RHIM domain is sufficient to serve as a pro-apoptotic adaptor and recruit RIPK1, FADD, and caspase-8 – thus leading to apoptosis.

Taken together, these examples clearly illustrate that RIPK3 forms an important component of host defense to an array of viral infections, as illustrated by the increased susceptibility of RIPK3-deficient mice. Many of these studies have taken as a starting point the canonical role of RIPK3 in triggering necroptosis, and have led to the idea that this form of cell death can eliminate the replicative niche and promote antiviral immunity. However, emerging data—including the demonstration of RIPK3-dependent apoptosis as a contributor to host defense against IAV—indicate that necrosome-mediated signaling is not limited to necroptosis. Thus, while RIPK3 mediates defense against viral infection, the nature of the signaling required for this defense remains the subject of ongoing investigation.

2.6 RIPK3 AND NECROPTOSIS IN THE CONTEXT OF BACTERIAL INFECTION

Cell death signaling also contributes to the immune response to bacterial infections, although the role of RIPK3 and necroptosis in these contexts is less clear. Many bacteria encode inhibitors of apoptotic signaling, such as NleB1 from enteropathogenic *E. coli* (EPEC), that block caspase activation and apoptosis in infected cells (68). This inhibition may prime infected cells for RIPK3-dependent necroptosis and elimination of the cellular space necessary for intracellular bacteria survival. However, the literature on RIPK3-depedent necroptosis and its contribution to antibacterial host defense is less conclusive, and probably highly context-dependent, as discussed below.

EPEC encodes a type III secretion system effector known as EspL, a cysteine protease that was recently shown to cleave the RHIM domains of RIPK1 and RIPK3, as well as TRIF and DAI, during infection—thus, inhibiting necroptosis and inflammation (69). Infection of WT mice with the EPEC-like pathogen *Citrobacter rodentium* results in intestinal colonization, which is attenuated in bacteria lacking EspL (69). This finding demonstrates the direct targeting of RHIM-dependent signaling by a bacterial effector protein, providing strong evidence that RHIM signaling contributes to host defense against bacterial pathogens. However, it remains unclear which aspects of RHIM-mediated signaling— which may include apoptotic, necroptotic, and inflammatory transcriptional signals— are at play in this context.

Staphylococcus aureus has been shown to activate RIPK3- and MLKL-mediated necroptosis. One study has reported that necroptosis contributes to bacterial clearance from infected skin, in part by eliminating cells that contribute to pathological inflammation (70). Interestingly, while MLKL-deficient mice have high bacterial loads, RIPK3-deficient mice show increased bacterial clearance and decreased inflammation, which hints at a necroptosis-

independent function for RIPK3 in promoting bacterial infection. Likewise, in a pulmonary model of *S. aureus* infection, *Ripk3*$^{-/-}$ mice have reduced bacterial loads (71).

Another example where RIPK3 deficiency may be beneficial is in the context of *Salmonella enterica* serovar Typhimurium infection. *S.* Typhimurium is a gram-negative bacterium that can induce multiple forms of cell death in macrophages, including caspase-1-dependent pyroptosis and RIPK3-dependent necroptosis, most likely as a means to promote its own spread (72). Consistent with this idea, RIPK3-deficient mice were moderately protected from *S.* Typhimurium infection compared to their WT counterparts (73). Likewise, in a zebrafish model of infection by *Mycobacterium tuberculosis*, RIPK3-dependent necroptosis in macrophages was shown to contribute to the release and dissemination of bacteria (74). These findings stand in contrast to the bacterial inhibition of RHIM-dependent necroptosis and inflammation exerted by EPEC, and highlight the fact that roles for these pathways in bacterial infection vary among pathogens and host tissues.

2.7 CONTRIBUTIONS OF RIPK3 TO TISSUE DAMAGE

As discussed, there is clear evidence that RIPK3 signaling— necroptotic or otherwise— can contribute to host defense against viral infection. However, the inflammatory and cell death responses triggered by RIPK3 represent a double-edged sword, and their aberrant activation may contribute to tissue pathology. Consistent with this idea, there is accumulating evidence that acute or chronic organ injury can lead to RIPK3-dependent contributions to the pathophysiology of a variety of diseases (reviewed in (75)). Interestingly, evidence from knockout mice has highlighted the differing contributions of MLKL and RIPK3 to these pathologies, supporting the idea that non-necroptotic functions of RIPK3 may underlie some of the observed effects.

RIPK3 signaling has been shown to be a critical contributor to damage in ischemia/reperfusion injury (IRI) in a variety of settings, including neonatal-hypoxia ischemic brain injury (76, 77), ischemic stroke (78), renal ischemia-reperfusion injury (IRI) (79, 80), and in cardiac ischemia and infarction (81-83). Implication of RIPK3 in these models led to the idea that necroptotic cell death contributes to tissue damage; however, more recent studies on MLKL knockout mice, in which necroptosis is deficient but other aspects of RIPK3 signaling persist, has led to a reevaluation of this idea. In 2012, Linkermann *et al.* used a high mortality model of renal ischemia to demonstrate that administration of the RIPK1 inhibitor Nec-1 to mice prior to renal IRI prolonged survival compared phosphate-buffered saline (PBS)-treated controls (80). Later, it was further demonstrated that catalytically inactive RIPK1 knock-in (D138N) mice, or RIPK3-deficient mice, were protected in a model of kidney IRI (84). Interestingly, however, MLKL deficiency did not afford mice the same level of protection in the kidney IRI model, which implies that there are other roles of RIPK3 beyond necroptotic cell death at work in this model (84).

Similarly, using a mouse model of myocardial infarction (MI), Luedde *et al.* demonstrated that RIPK3 is upregulated and can lead to necroptosis after MI. In addition, they showed that RIPK3-deficient mice have diminished inflammatory responses and reactive oxygen species (ROS) production in infarcted hearts compared to their WT counterparts (83). These data were further verified by Newton *et al.* in 2016 (84). However, these studies did not include a comparison with MLKL-deficient mice, so it is difficult to conclude whether the observed damage was mediated by MLKL or by RIPK3 transcriptional and inflammatory responses.

Yet another example of detrimental RIPK3-dependent signaling is the neurodegenerative disease amyotrophic lateral sclerosis (ALS). Loss of the protein optineurin (Optn), which has

been implicated in ALS, sensitizes glial cells of the CNS to necroptosis, leading to demyelination and axonal degeneration (85). This is clearly demonstrated in mouse embryonic fibroblasts (MEFs) derived from *Optn*$^{-/-}$ mice, as well as in the spinal cords of *Optn*$^{-/-}$ mice, which have increased levels of complex-associated RIPK1, RIPK3, and phospho-MLKL, which can be used as a marker of necroptosis. Interestingly, the axonal pathology and hindlimb weakness of Optn-deficient mice was rescued in *Optn*$^{-/-}$*Ripk1*$^{D138N/D138N}$ mice, as well as *Optn*$^{-/-}$*Ripk3*$^{-/-}$ double mutant mice, and treatment of *Optn*$^{-/-}$ mice with Nec-1s (a highly specific inhibitor of RIPK1 catalytic activity) led to a reduction of neuropathology (85). Together, these data suggest that uncontrolled RIPK3-dependent necroptosis can be harmful in the context of ALS, and have led to efforts to target this pathway pharmacologically in the clinic.

RIPK3-dependent death has also been reported to contribute to hepatocyte cell death from ethanol-induced injury (86). Liver biopsies from patients with alcoholic liver disease (ALD) showed higher RIPK3 expression compared with controls. RIPK3 was also shown to be upregulated in the livers of ethanol-fed mice, and RIPK3-deficient mice were significantly protected against ethanol-induced liver injury and inflammation (cytokine levels of MCP-1, IL-6, and TNFα) compared to WT mice. Interestingly, however, administration of Nec-1 did not reduce ethanol-induced hepatocyte injury, as measured by plasma ALT/AST levels (86). This may indicate that RIPK3-dependent necroptosis proceeds independent of RIPK1 kinase activity in this setting, though other functions of RIPK3 signaling could also contribute to this pathology.

These examples illustrate that in situations of tissue injury, RIPK3 activation can lead to detrimental effects in the host. However, in several of the settings highlighted above, it has emerged that the observed effects are RIPK3-dependent but MLKL-independent, implicating non-death functions of the necrosome in the observed pathology. Additional studies, including

work with newly-available mouse models in which specific functional domains of RIPK1 and/or RIPK3 are mutated, will provide insight into the nature of the signals driving tissue damage in these settings.

2.8 THE ROLE OF RIPK3 IN INFLAMMATION

While RIPK3 is best known for its role in cell death, the findings discussed above— and in particular the distinct phenotypes observed between *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ animals in both viral infection and sterile tissue pathology—has made it clear that RIPK3 participates in additional signaling pathways. The question of how RIPK3 signaling and necroptosis participate in host immune responses and immunopathology hinges on how necrosome signaling contributes to inflammation and the initiation of innate immune responses. In turn, this question can be roughly divided into two sub-topics. First, how does necroptosis contribute to inflammation? And second, what non-necroptotic signals— such as activation of inflammatory transcription pathways— also emanate from the necrosome? These two questions clearly bear on each other: cell death would be predicted to terminate inflammatory transcription, and targets of inflammatory transcription may inhibit cell death. Having highlighted physiological settings in which RIPK3 signaling is important, we will now consider evidence for the pathways mediated by RIPK3, including both necroptotic and transcriptional effects.

Because necroptosis is a lytic form of cell death, it is thought that the release of danger associated molecular patterns (DAMPs) from necroptotic cells contributes to inflammation. Examples of DAMPs include intracellular components such as the chromatin-associated protein high-mobility group box 1 (HMGB-1), heat shock proteins (HSPs), F-actin, as well as DNA, RNA, and ATP; as these molecules are normally contained within cells, their release may be sensed as a signature of tissue damage, leading to activation of local innate immune cells and

thus to inflammation (2). It is thought that bystander cells sense these molecules and events through the same PRRs that sense pathogen-associated molecular patterns (PAMPs) or through specialized DAMP receptors, and initiate an inflammatory response. Thus, necroptotic cell death has been considered pro-inflammatory due to DAMP release.

An example that hints at the importance of DAMPs from necroptotic cells comes from an *in vivo* model of TNF-induced toxicity, systemic inflammatory response syndrome (SIRS). When *Ripk3*$^{-/-}$ mice are treated with high doses of TNF, they fare better than their WT counterparts in terms of both morbidity and mortality (87). Likewise, pre-treatment of WT mice with Nec-1 or use of mice lacking the kinase activity of RIPK1 provides similar protection to high doses of TNF (50, 84, 87). This suggests that necroptosis mediates cellular damage that eventually leads to mortality; in fact, levels of certain DAMPs and cytokines are reduced in RIPK3-deficient mice in this model (87). This finding is in keeping with the idea that lytic cell death and resultant inflammation and tissue damage are a key outcome of RIPK3 signaling. However, another study found that loss of MLKL was not as effective as RIPK3-deficiency in the high-dose TNF model (84), suggesting that RIPK3 and RIPK1 may be regulating more than simply MLKL-dependent death.

In keeping with the notion that RIPK3 controls more than just necroptotic death, RIPK3 has also been implicated in the activation of inflammatory transcription. Using transient overexpression systems, early studies of RIPK1 and RIPK3 found that while RIPK1 potently activated NF-κB porter genes, RIPK3 was comparatively less effective, and could even inhibit TNF-induced NF-κB activation (88-90). Nonetheless, RIPK3 was also identified in a screen for proteins that could activate an NF-κB reporter gene (91). In 2004, Newton *et al.* showed that RIPK3-deficient cells did not differ in their ability to engage NF-κB signaling in response to

TNF, LPS, peptidoglycan, or downstream of B- or T- cell antigen receptor crosslinking (52). As we now appreciate RIPK3's role in cell death, early studies demonstrating that RIPK3 has an inhibitory role in RIPK1- or TNF-induced NF-κB activation can now be revisited with the lens of necroptosis— in fact, it makes sense that RIPK3 overexpression or activation would dampen NF-κB responses, as cell death would cause termination of biological processes. In keeping with this view, it was recently shown that necroptosis leads to the suppression of TNF- or LPS-induced inflammation by ceasing cytokine and chemokine production (92).

However, somewhat contrary to the idea that necroptotic death terminates inflammation, there is evidence that RIPK3 activation can lead to *de novo* synthesis of pro-inflammatory cytokines and chemokines. Using a system of direct RIPK3 activation, independent of upstream receptor signaling, Yatim *et al.* demonstrated that chemically-enforced RIPK3 oligomerization leads to the recruitment of RIPK1 and subsequent NF-κB activation (93). The authors went on to demonstrate that this NF-κB signaling in necroptotic cells determines the ability of dendritic cells (DCs) to cross-prime CD8+ T cells with antigens derived from the dying cells. Further, a more recent study showed that the kinase activity of RIPK1, as well as RIPK3 itself, contributed to TRIF-dependent inflammatory transcription upon TLR4 activation in bone marrow-derived macrophages (94). RIPK1 has well-described roles in NF-κB activation, but these canonical functions are independent of the kinase activity of RIPK1, or of RIPK3 engagement (reviewed in (95)). How RIPK3 activity and necrosome formation contribute to NF-κB or other transcriptional pathways is still poorly understood.

Moreover, there is evidence that RIPK3 leads to cytokine expression downstream of injury and promotes tissue repair. In a 2014 publication, Moriwaki *et al.* demonstrated that surprisingly, the presence of RIPK3 protects against dextran sodium sulfate (DSS)-induced

colitis, and that RIPK3 is required for tissue repair by inducing an axis of IL-23, IL-1β and IL-22 downstream of DSS-induced injury (96). However, contrary to these results, another study found that loss of RIPK3 had no effect on DSS-induced colitis (84). Different DSS-administration and recovery protocols, as well as duration of health monitoring, and differences in colony microbiota between institutions, might contribute to the differences observed. Nonetheless, it is interesting to speculate that RIPK3 may play a role in promoting tissue injury (or repair) and inflammation, independent of its role in cell death.

Genetic models have highlighted RIPK3-dependent inflammation that both does and does not depend on MLKL-dependent cell death. Most clearly, loss of caspase-8 causes embryonic lethality in mice, which is rescued by co-ablation of either RIPK3 or MLKL, supporting the notion that it is unchecked MLKL-dependent cell death, and not some other function of RIPK3, that leads to lethality in *Casp8*$^{-/-}$ animals. However, *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ double-knockout mice display a phenotype somewhat distinct from that observed in *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ double knockout animals, including an accelerated accumulation of aberrant lymphocyte populations and increased inflammatory cytokines in the serum (29). These differences may reflect death-independent RIPK3 signaling, which would still be intact in *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ animals. Other models also highlight the dichotomy of death-dependent and independent functions for RIPK3. Skin inflammation due to RIPK1 ablation can be ameliorated in mice by deleting either RIPK3 or MLKL, supporting a role for MLKL-dependent cell death in this setting (97). However, in other genetic models of inflammation this does not hold true. A20 (also known as TNFAIP3) is a pro-survival protein, and A20-deficient mice suffer systemic inflammation and die shortly after birth (98). Interestingly, while RIPK3 deficiency or catalytically inactive RIPK1 (*Ripk1*$^{KD/KD}$) delay the mortality of *A20*$^{-/-}$ mice, MLKL deficiency does not (84). This indicates that MLKL-

dependent necroptosis does not contribute significantly to the morbidity and mortality associated with A20 deficiency; instead, other as-yet-unclear roles of necrosome signaling appear to mediate these effects.

While it is now appreciated that RIPK3 can act in concert with RIPK1 to engage in pro-inflammatory, non-cell death signaling, proteins that regulate the necrosome, such as caspase-8 and cFLIP, may also be involved in inflammatory processes. Recent work has demonstrated an unexpected, cell-intrinsic role for caspase-8 in the initiation of inflammatory transcription, in addition to its functions in initiation apoptosis and suppressing necroptosis. This was shown to be important during the host response to *Yersinia* and *Salmonella* infection, as well as downstream of direct activation of TLR4 by LPS (99). The mechanism by which caspase-8 activation leads to upregulation of pro-inflammatory cytokines is not clear, although it is known that caspase-8 enzymatic activity is necessary, and a recent publication implicated the caspase-8/cFLIP heterodimer as the species involved, by showing that a version of caspase-8 that cannot undergo interdomain cleavage was able to support the transcriptional, but not apoptotic, functions of this enzyme. It is intriguing that this caspase-8/cFLIP heterodimer is also the complex that is recruited to the necrosome to suppress necroptosis (99). Future work will undoubtedly focus on putative roles for necrosome formation in caspase-8-dependent transcriptional pathways.

The RIP kinases and caspase-8 can contribute to inflammation via non-transcriptional mechanisms, as well. Interestingly, one such example is in the case of inflammasome activation and the processing of pro-IL-1β. While pro-IL-1β is an inflammatory cytokine that is canonically cleaved— and thereby activated— by caspase-1, under certain conditions it can be cleaved by caspase-8 (reviewed in (100)) . Moreover, RIPK3 can promote IL-1β processing and secretion in

response to LPS stimulation by assembling a complex including caspase-8, FADD, RIPK1, and requiring TRIF, in macrophages and DCs (101). The kinase activity of RIPK1 and RIPK3 is not required for this atypical non-apoptotic caspase-8 activation and in fact, treatment of cells with a RIPK3 kinase inhibitor enhances LPS-induced caspase-8 activation and thus IL-1β processing (101). Furthermore, recent studies have demonstrated an additional link between necroptosis in inflammasome activation, by showing that MLKL activation, which leads to membrane disruption, can activate the NLRP3 inflammasome in a cell-intrinsic manner by altering ion homeostasis (102, 103). This finding implies that induction of necroptosis is linked inextricably to inflammasome assembly, and that caspase-1 activation and processing of IL-1β and IL-18 likely accompany necroptotic cell death when all are present.

So what can we conclude from these complex and sometimes contradictory results? It seems clear that necroptosis can occur physiologically, and that in some settings this can contribute to host defense. It also seems apparent that non-necroptotic functions of RIPK1 and RIPK3 are important contributors to both beneficial immune responses and pathophysiology, though the precise nature of these signals remain to be elucidated.

2.9 CONCLUDING REMARKS

RIPK3 has traditionally been considered a pro-necrotic protein, although we now appreciate that it has non-necroptotic roles in a variety of physiological settings. From an evolutionary perspective, it may be that RIPK3 evolved as a pro-inflammatory protein alongside its homolog RIPK1, and this pathway was re-purposed to induce inflammatory death upon caspase-8 inhibition or absence (reviewed in (104)). Because programmed necrosis can occur in response to pathogen infection, it follows that perhaps this form of cell death evolved as a

"backup" to apoptosis when pathogens themselves evolved mechanisms to inhibit caspase-8. Whether this is the case, however, remains an open question.

Furthermore, separating RIPK3 signaling from caspase-8 and RIPK1 remains challenging. RIPK1 has been shown to play a crucial role in promoting NF-κB activation and inflammation downstream of TNFR1 signaling, and recently was shown to be important for the inflammation and cross-priming potential of necroptotic cells after direct RIPK3 activation. Caspase-8 also has distinct transcriptional and post-translational roles in inflammation. As both RIPK1 and caspase-8 can promote inflammation, it will be important to elucidate RIPK3's role in inflammation both independent of and in concert with these other proteins. Further, persistent questions remain about the relative contributions of MLKL-dependent necroptosis and non-death functions of RIPK3 during host defense and tissue damage. Studies comparing RIPK3-deficient and MLKL-deficient mice in a variety of disease models will be necessary to elucidate the contribution of necroptosis versus RIPK3 non-death signaling.

2.10 Acknowledgements and funding

We thank Michelle Brault and Dr. Brian Daniels for editorial comments. This work is supported by NIH grants 3R01AI108685-01S1 (SO), 1R01AI108685-01 and 1R21CA185681 (AO).

2.11 References

1. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science.1998;281:1312-1316.

2. Kono H, Rock KL. How dying cells alert the immune system to danger. Nature reviews Immunology.2008;8:279-289.

3. Vanden Berghe T, et al. Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. Cell death and differentiation.2010;17:922-930.

4. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature reviews Molecular cell biology.2010;11:700-714.

5. Degterev A, et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol.2008;4:313-321.

6. Lin Y, et al. Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. The Journal of biological chemistry.2004;279:10822-10828.

7. Cho YS, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell.2009;137:1112-1123.

8. He S, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell.2009;137:1100-1111.

9. Zhang DW, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science.2009;325:332-336.

10. Linkermann A, Green DR. Necroptosis. N Engl J Med.2014;370:455-465.

11. Chan FK, Luz NF, Moriwaki K. Programmed necrosis in the cross talk of cell death and inflammation. Annu Rev Immunol.2015;33:79-106.

12. Newton K, Manning G. Necroptosis and Inflammation. Annu Rev Biochem.2016;85:743-763.

13. Li J, et al. The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell.2012;150:339-350.

14. Feoktistova M, et al. cIAPs Block Ripoptosome Formation, a RIP1/Caspase-8 Containing Intracellular Cell Death Complex Differentially Regulated by cFLIP Isoforms. Molecular cell.2011.

15. Geserick P, et al. Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. J Cell Biol.2009;187:1037-1054.

16. Oberst A, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature.2011;471:363-367.

17. Orozco S, et al. RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. Cell death and differentiation.2014;21:1511-1521.

18. Kaiser WJ, et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature.2011;471:368-372.

19. Sun L, et al. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell.2012;148:213-227.

20. Zhao J, et al. Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proceedings of the National Academy of Sciences of the United States of America.2012;109:5322-5327.

21. Chen W, et al. Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling. The Journal of biological chemistry.2013;288:16247-16261.

22. Wu J, et al. Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. Cell Res.2013;23:994-1006.

23. Murphy JM, et al. The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. Immunity.2013;39:443-453.

24. Hildebrand JM, et al. Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. Proceedings of the National Academy of Sciences of the United States of America.2014;111:15072-15077.

25. Cai Z, et al. Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. Nat Cell Biol.2014;16:55-65.

26. Chen X, et al. Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. Cell Res.2014;24:105-121.

27. Wang H, et al. Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. Mol Cell.2014;54:133-146.

28. Dondelinger Y, et al. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. Cell Rep.2014;7:971-981.

29. Alvarez-Diaz S, et al. The Pseudokinase MLKL and the Kinase RIPK3 Have Distinct Roles in Autoimmune Disease Caused by Loss of Death-Receptor-Induced Apoptosis. Immunity.2016;45:513-526.

30. Kaiser WJ, Upton JW, Mocarski ES. Viral modulation of programmed necrosis. Curr Opin Virol.2013;3:296-306.

31. Ofengeim D, Yuan J. Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. Nat Rev Mol Cell Biol.2013;14:727-736.

32. Wilson NS, Dixit V, Ashkenazi A. Death receptor signal transducers: nodes of coordination in immune signaling networks. Nat Immunol.2009;10:348-355.

33. Ting AT, Pimentel-Muinos FX, Seed B. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. EMBO J.1996;15:6189-6196.

34. Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity.1996;4:387-396.

35. Kim JW, Joe CO, Choi EJ. Role of receptor-interacting protein in tumor necrosis factor-alpha -dependent MEKK1 activation. The Journal of biological chemistry.2001;276:27064-27070.

36. Yang J, et al. The essential role of MEKK3 in TNF-induced NF-kappaB activation. Nat Immunol.2001;2:620-624.

37. Kreuz S, Siegmund D, Scheurich P, Wajant H. NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol.2001;21:3964-3973.

38. Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J. NF-kappaB signals induce the expression of c-FLIP. Mol Cell Biol.2001;21:5299-5305.

39. He S, Liang Y, Shao F, Wang X. Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proceedings of the National Academy of Sciences of the United States of America.2011;108:20054-20059.

40. Kaiser WJ, et al. Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. The Journal of biological chemistry.2013;288:31268-31279.

41. Ullah MO, Sweet MJ, Mansell A, Kellie S, Kobe B. TRIF-dependent TLR signaling, its functions in host defense and inflammation, and its potential as a therapeutic target. J Leukoc Biol.2016;100:27-45.

42. Meylan E, et al. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol.2004;5:503-507.

43. Takaoka A, et al. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature.2007;448:501-505.

44. Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell host & microbe.2012;11:290-297.

45. Rebsamen M, et al. DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF-kappaB. EMBO reports.2009;10:916-922.

46. Kuriakose T, et al. ZBP1/DAI is an innate sensor of influenza virus triggering the NLRP3 inflammasome and programmed cell death pathways. Sci Immunol.2016;1.

47. Thapa RJ, et al. DAI Senses Influenza A Virus Genomic RNA and Activates RIPK3-Dependent Cell Death. Cell host & microbe.2016;20:674-681.

48. Newton K, et al. RIPK1 inhibits ZBP1-driven necroptosis during development. Nature.2016;540:129-133.

49. Lin J, et al. RIPK1 counteracts ZBP1-mediated necroptosis to inhibit inflammation. Nature.2016;540:124-128.

50. Newton K, et al. Activity of protein kinase RIPK3 determines whether cells die by necroptosis or apoptosis. Science.2014;343:1357-1360.

51. Mandal P, et al. RIP3 induces apoptosis independent of pronecrotic kinase activity. Mol Cell.2014;56:481-495.

52. Newton K, Sun X, Dixit VM. Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. Mol Cell Biol.2004;24:1464-1469.

53. Nogusa S, et al. RIPK3 Activates Parallel Pathways of MLKL-Driven Necroptosis and FADD-Mediated Apoptosis to Protect against Influenza A Virus. Cell host & microbe.2016;20:13-24.

54. Li M, Beg AA. Induction of necrotic-like cell death by tumor necrosis factor alpha and caspase inhibitors: novel mechanism for killing virus-infected cells. J Virol.2000;74:7470-7477.

55. Menard C, et al. Role of murine cytomegalovirus US22 gene family members in replication in macrophages. J Virol.2003;77:5557-5570.

56. Brune W, Menard C, Heesemann J, Koszinowski UH. A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. Science.2001;291:303-305.

57. Upton JW, Kaiser WJ, Mocarski ES. Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. The Journal of biological chemistry.2008;283:16966-16970.

58. Upton JW, Kaiser WJ, Mocarski ES. Virus inhibition of RIP3-dependent necrosis. Cell host & microbe.2010;7:302-313.

59. Lembo D, et al. The ribonucleotide reductase R1 homolog of murine cytomegalovirus is not a functional enzyme subunit but is required for pathogenesis. J Virol.2004;78:4278-4288.

60. Skaletskaya A, Bartle LM, Chittenden T, McCormick AL, Mocarski ES, Goldmacher VS. A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. Proceedings of the National Academy of Sciences of the United States of America.2001;98:7829-7834.

61. Omoto S, Guo H, Talekar GR, Roback L, Kaiser WJ, Mocarski ES. Suppression of RIP3-dependent necroptosis by human cytomegalovirus. The Journal of biological chemistry.2015;290:11635-11648.

62. Galvan V, Roizman B. Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exogenous inducers in a cell-type-dependent manner. Proceedings of the National Academy of Sciences of the United States of America.1998;95:3931-3936.

63. Leopardi R, Van Sant C, Roizman B. The herpes simplex virus 1 protein kinase US3 is required for protection from apoptosis induced by the virus. Proceedings of the National Academy of Sciences of the United States of America.1997;94:7891-7896.

64. Guo H, et al. Herpes simplex virus suppresses necroptosis in human cells. Cell host & microbe.2015;17:243-251.

65. Huang Z, et al. RIP1/RIP3 binding to HSV-1 ICP6 initiates necroptosis to restrict virus propagation in mice. Cell host & microbe.2015;17:229-242.

66. Rodrigue-Gervais IG, et al. Cellular inhibitor of apoptosis protein cIAP2 protects against pulmonary tissue necrosis during influenza virus infection to promote host survival. Cell host & microbe.2014;15:23-35.

67. Harris KG, et al. RIP3 Regulates Autophagy and Promotes Coxsackievirus B3 Infection of Intestinal Epithelial Cells. Cell host & microbe.2015;18:221-232.

68. Sridharan H, Upton JW. Programmed necrosis in microbial pathogenesis. Trends Microbiol.2014;22:199-207.

69. Pearson JS, et al. EspL is a bacterial cysteine protease effector that cleaves RHIM proteins to block necroptosis and inflammation. Nat Microbiol.2017;2:16258.

70. Kitur K, et al. Necroptosis Promotes Staphylococcus aureus Clearance by Inhibiting Excessive Inflammatory Signaling. Cell Rep.2016;16:2219-2230.

71. Kitur K, et al. Toxin-induced necroptosis is a major mechanism of Staphylococcus aureus lung damage. PLoS Pathog.2015;11:e1004820.

72. Hu ZQ, Zhao WH. Type 1 interferon-associated necroptosis: a novel mechanism for Salmonella enterica Typhimurium to induce macrophage death. Cell Mol Immunol.2013;10:10-12.

73. Robinson N, McComb S, Mulligan R, Dudani R, Krishnan L, Sad S. Type I interferon induces necroptosis in macrophages during infection with Salmonella enterica serovar Typhimurium. Nat Immunol.2012;13:954-962.

74. Roca FJ, Ramakrishnan L. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. Cell.2013;153:521-534.

75. Zhao H, Jaffer T, Eguchi S, Wang Z, Linkermann A, Ma D. Role of necroptosis in the pathogenesis of solid organ injury. Cell Death Dis.2015;6:e1975.

76. Chavez-Valdez R, Martin LJ, Northington FJ. Programmed Necrosis: A Prominent Mechanism of Cell Death following Neonatal Brain Injury. Neurol Res Int.2012;2012:257563.

77. Northington FJ, Chavez-Valdez R, Graham EM, Razdan S, Gauda EB, Martin LJ. Necrostatin decreases oxidative damage, inflammation, and injury after neonatal HI. J Cereb Blood Flow Metab.2011;31:178-189.

78. Degterev A, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol.2005;1:112-119.

79. Linkermann A, Chen G, Dong G, Kunzendorf U, Krautwald S, Dong Z. Regulated cell death in AKI. J Am Soc Nephrol.2014;25:2689-2701.

80. Linkermann A, et al. Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. Kidney Int.2012;81:751-761.

81. Koshinuma S, Miyamae M, Kaneda K, Kotani J, Figueredo VM. Combination of necroptosis and apoptosis inhibition enhances cardioprotection against myocardial ischemia-reperfusion injury. J Anesth.2014;28:235-241.

82. Oerlemans MI, et al. Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo. Basic Res Cardiol.2012;107:270.

83. Luedde M, et al. RIP3, a kinase promoting necroptotic cell death, mediates adverse remodelling after myocardial infarction. Cardiovasc Res.2014;103:206-216.

84. Newton K, et al. RIPK3 deficiency or catalytically inactive RIPK1 provides greater benefit than MLKL deficiency in mouse models of inflammation and tissue injury. Cell death and differentiation.2016;23:1565-1576.

85. Ito Y, et al. RIPK1 mediates axonal degeneration by promoting inflammation and necroptosis in ALS. Science.2016;353:603-608.

86. Roychowdhury S, McMullen MR, Pisano SG, Liu X, Nagy LE. Absence of receptor interacting protein kinase 3 prevents ethanol-induced liver injury. Hepatology.2013;57:1773-1783.

87. Duprez L, et al. RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity.2011;35:908-918.

88. Kasof GM, Prosser JC, Liu D, Lorenzi MV, Gomes BC. The RIP-like kinase, RIP3, induces apoptosis and NF-kappaB nuclear translocation and localizes to mitochondria. FEBS Lett.2000;473:285-291.

89. Yu PW, et al. Identification of RIP3, a RIP-like kinase that activates apoptosis and NFkappaB. Curr Biol.1999;9:539-542.

90. Sun X, Lee J, Navas T, Baldwin DT, Stewart TA, Dixit VM. RIP3, a novel apoptosis-inducing kinase. The Journal of biological chemistry.1999;274:16871-16875.

91. Pomerantz JL, Denny EM, Baltimore D. CARD11 mediates factor-specific activation of NF-kappaB by the T cell receptor complex. EMBO J.2002;21:5184-5194.

92. Kearney CJ, et al. Necroptosis suppresses inflammation via termination of TNF- or LPS-induced cytokine and chemokine production. Cell death and differentiation.2015;22:1313-1327.

93. Yatim N, et al. RIPK1 and NF-kappaB signaling in dying cells determines cross-priming of CD8(+) T cells. Science.2015;350:328-334.

94. Najjar M, et al. RIPK1 and RIPK3 Kinases Promote Cell-Death-Independent Inflammation by Toll-like Receptor 4. Immunity.2016;45:46-59.

95. Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P. RIP1, a kinase on the crossroads of a cell's decision to live or die. Cell death and differentiation.2007;14:400-410.

96. Moriwaki K, Balaji S, McQuade T, Malhotra N, Kang J, Chan FK. The necroptosis adaptor RIPK3 promotes injury-induced cytokine expression and tissue repair. Immunity.2014;41:567-578.

97. Dannappel M, et al. RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. Nature.2014;513:90-94.

98. Lee EG, et al. Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science.2000;289:2350-2354.

99. Philip NH, et al. Activity of Uncleaved Caspase-8 Controls Anti-bacterial Immune Defense and TLR-Induced Cytokine Production Independent of Cell Death. PLoS Pathog.2016;12:e1005910.

100. Gurung P, Kanneganti TD. Novel roles for caspase-8 in IL-1beta and inflammasome regulation. Am J Pathol.2015;185:17-25.

101. Moriwaki K, Bertin J, Gough PJ, Chan FK. A RIPK3-caspase 8 complex mediates atypical pro-IL-1beta processing. J Immunol.2015;194:1938-1944.

102. Gutierrez KD, et al. MLKL Activation Triggers NLRP3-Mediated Processing and Release of IL-1beta Independently of Gasdermin-D. J Immunol.2017.

103. Conos SA, et al. Active MLKL triggers the NLRP3 inflammasome in a cell-intrinsic manner. Proceedings of the National Academy of Sciences of the United States of America.2017;114:E961-E969.

104. Brault M, Oberst A. Controlled detonation: evolution of necroptosis in pathogen defense. Immunol Cell Biol.2016.

Chapter 3. RIPK1 BOTH POSITIVELY AND NEGATIVELY REGULATES RIPK3 OLIGOMERIZATION AND NECROPTOSIS

This chapter is based on the following publication:

Orozco S, *et al*. RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. *Cell Death and Differentiation.* 2014;21:1511-1521.
https://www.ncbi.nlm.nih.gov/pubmed/24902904

3.1 SUMMARY

Necroptosis is a form of programmed cell death that depends on the activation of Receptor Interacting Protein Kinases-1 (RIPK1) and RIPK3 by receptors such as TNFR1. Structural studies indicate that activation of RIPK3 by RIPK1 involves the formation of oligomers via interactions of the RIP homotypic interaction motif (RHIM) domains shared by both proteins; however, the molecular mechanisms by which this occurs are not fully understood. To gain insight into this process, we constructed versions of RIPK3 that could be induced to dimerize or oligomerize in response to a synthetic drug. Using this system, we find that while the formation of RIPK3 dimers is itself insufficient to trigger cell death, this dimerization seeds a RHIM-dependent complex, the propagation and stability of which is controlled by caspase-8 and RIPK1. Consistent with this idea, we find that chemically-enforced oligomerization of RIPK3 is sufficient to induce necroptosis, independent of the presence of the RHIM domain, TNF stimulation or RIPK1 activity. Further, while RIPK1 contributes to TNF-mediated RIPK3 activation, we find that the RIPK1 protein intrinsically suppresses spontaneous RIPK3 activation in the cytosol by controlling RIPK3 oligomerization. Cells lacking RIPK1 protein undergo increased spontaneous RIPK3-dependent death upon accumulation of the RIPK3 protein, while cells containing a chemically inhibited form of RIPK1 were protected from this form of death. Together, these data indicate that RIPK1 can activate RIPK3 in response to receptor signaling, but also acts as a negative regulator of spontaneous RIPK3 activation in the cytosol.

3.2 INTRODUCTION

Necroptosis is a form of programmed cell death that is both mechanistically and morphologically distinct from apoptosis.[1,2] Although apoptosis is defined by the activation of the

caspase proteases, necroptosis is triggered by receptor-interacting protein kinase 1 (RIPK1 (Degterev et al.[3] and Lin et al.[4]) and RIPK3.[5–7] Morphologically, necroptosis resembles the unprogrammed process of necrosis, involving cellular swelling and rupture.8 This morphology is distinct from apoptosis, in which dying cells shrink and their contents remain contained within membrane-bound bodies or vesicles. Necroptotic cell death thereby releases cellular contents that are contained during apoptosis; necroptosis is therefore thought to be an inflammatory form of cell death. Consistent with a proposed role in inflammation and immune responses, necroptosis can be triggered by tumor necrosis factor (TNF),[2] interferon[9] or Toll-like receptor[10] signaling, as well as by viral infection via the DNA sensor DAI (DNA-dependent activator of interferon regulatory factor).[11] Necroptotic cell death has a role in the host response to viral and bacterial infection,[5,11–13] as well as the pathogenesis of TNF-induced sterile septic shock.[14] The mechanism by which the necroptotic program is initiated has been studied principally in the context of TNF receptor-1 (TNFR1) activation, and it remains incompletely understood. Briefly, ligation of TNFR1 by TNF induces the assembly of a large receptor-proximal complex that includes RIPK1.[15] Ubiquitination and phosphorylation events within this complex lead to activation of an nuclear factor-kB transcriptional program and/or MAP kinase activation.[15] Subsequently, RIPK1 is deubiquitinated and translocates into the cytosol,[16] where it forms additional complexes that have been termed ‘necrosomes’[17] or ‘ripoptosomes’;[18,19] these scaffolds support RIPK3 activation, which in turn leads to phosphorylation of the downstream mediator mixed-lineage kinase-like (MLKL)[20–23] and the process of necroptosis. Importantly, the cIAP ubiquitin ligases[18,24] and the pro-apoptotic enzyme caspase-8,[25,26] in concert with its paralog cFLIPL (cellular flice-like inhibitory protein, long isoform), can also be recruited to necrosome complexes, and they antagonize RIPK3 activation and necroptosis. The assembly and

regulation of the RIPK1–RIPK3 necrosome is an open subject of investigation in the field. Recent structural analysis showed that the RIP homotypic interaction motif (RHIM) domains of RIPK1 and RIPK3 form amyloid-like oligomers during RIPK3 activation;[17] however, it remains unclear whether RIPK3 oligomerization, RHIM amyloid formation, or both are necessary and/or sufficient for RIPK3 activation. Furthermore, it is unclear how suppressors of necroptosis, such as caspase-8, interact with and regulate RIPK3 oligomers to determine cell fate. Inducible protein interaction systems have provided fundamental insight into many cellular processes, including cell death. For example, we and others have used versions of the FK506-binding protein (FKBP)–rapamycin interaction system[27] to create caspase proteases that could be induced to undergo homo- or heterodimerization by addition of specific drug ligands.[28–31] Here we applied similar strategies to the study of RIPK3, with the goal of defining how its activation is regulated during cell life and in response to stress events that culminate in the induction of necroptosis. Using these systems, we found that dimerization of RIPK3 is able to seed a RHIM-dependent oligomer, the propagation of which is required for induction of necroptosis. This RHIM-dependent oligomerization is directly regulated by RIPK1 and caspase-8. Unexpectedly, we found that although chemical inhibition of RIPK1 inhibited RHIM-dependent RIPK3 oligomerization and cell death, depletion of RIPK1 protein in this system had the opposite effect. Together, these data indicate that RIPK3 oligomerization is both necessary and sufficient for the induction of necroptosis, and that RHIM-dependent oligomerization of RIPK3 recruits capase-8 and RIPK1, which control this process. Further, although RIPK1 is required for receptorinduced activation of RIPK3, we show that it also exerts intrinsic suppression of RIPK3 oligomerization in the cytosol.

3.3 RESULTS

RIPK3 dimerization triggers necroptosis, which requires the RHIM domain of RIPK3.

In an effort to gain insight into the mechanism by which RIPK3 is activated, we created a chimeric protein composed of murine RIPK3 fused to a single copy of $FKBP^{F36V}$ (hereafter '$FKBP^{F36V}$ point mutant (FV) domain'), a protein domain that binds with high affinity to a synthetic bivalent homologue of rapamycin, here called 'AP1' (homodimerization drug; Figure 1a). FV domains rapidly dimerize in response to AP1 treatment,[27] and we took advantage of this property to investigate the protein–protein interactions involved in RIPK3 activation and necroptosis. Importantly, we chose to append the FV domain to the C-terminus of RIPK3, the same position at which the RHIM domain is located, in an effort to faithfully mimic RHIM-dependent interactions that define RIPK3 activation.[17] We expressed RIPK3-1xFV (Figure 1a) in NIH-3T3 cells, a cell line that lacks endogenous RIPK3 expression and is therefore unresponsive to TNF-induced necroptosis (Supplementary Figures S1A and B). We then quantified the cell death responses of NIH-3T3 cells expressing our constructs over time using the IncuCyte imaging system, which allows precise quantification of cell death with high temporal resolution (Supplementary Movies 1 and 2). Expression of RIPK3-1xFV sensitized these cells to necroptosis induced by the combination of TNF and the caspase inhibitor Z-val-ala-asp-(O-methlylated)-fluoromethylketone (zVAD) in a manner analogous to that observed in Jax cells, a murine fibroblast line that expresses endogenous RIPK3 (Supplementary Figure S1C compared with Supplementary Figure S1A). These data indicate that our construct could be faithfully activated by the well-described pathway of TNF receptor-driven cell death. Next, we added AP1 to these cells in the absence of TNF, to test the effect of chemically enforced RIPK3 dimerization in the cytosol. We found that on AP1 addition, these cells underwent limited RIPK3

activation and necroptosis (Figure 1b) in a manner that depended on the concentration of AP1 added. To ensure that the cell death observed on dimerizer addition was not influenced by autocrine TNF production in our cells, we treated them with the TNF blocking reagent TNFR1–immunoglobulin Fc fusion protein (TNFR-Fc). Although TNFR-Fc efficiently inhibited TNF-induced necroptosis in these cells (Supplementary Figure S1D), it did not affect AP1-induced cell death (Figure 1c). However, dimerizer-induced cell death did require the kinase activity of RIPK3 (Supplementary Figure S1E), as well as the downstream mediator of necroptosis MLKL (Figure 1e and Supplementary Figure S2A). Mutation of key phosphorylation sites required for interaction between RIPK3 and MLKL20 also rendered RIPK3-1xFV unable to induce cell death on either TNF or AP1 treatment (Supplementary Figures S1F and I). Together, these data confirm that RIPK3 dimerization leads to necroptosis via direct activation of RIPK3. The RIP kinases interact via RHIM domains, and mutation of this domain in RIPK3 renders it unresponsive to receptor-driven necroptosis (Supplementary Figure S1G and Li et al.[17]). We next tested the requirement for this domain in RIPK3 activation via dimerization. To our surprise, given that we were inducing RIPK3 interaction via AP1-mediated homodimerization, we found that versions RIPK3-1xFV in which the RHIM domain was deleted (RIPK3DC-1xFV), or in which the key RHIM amino acid sequence VQIG was modified to AAAA (RIPK3DRHIM-1xFV) failed to trigger cell death following AP1 treatment (Figure 1d and Supplementary Figures S1G–I). Recent structural evidence demonstrated that the RHIM domain of RIPK3 forms amyloid-like oligomers during RIPK3 activation.[17] We therefore hypothesized that although RIPK3 dimerization itself is insufficient for its activation, it may ‘seed’ RHIM oligomers that recruit both RIPK1 and additional molecules of RIPK3, and whose propagation allows RIPK3 activation. To directly test this idea and capture evidence of RIPK3 dimers or oligomers, we

performed DSS crosslinking experiments on cells expressing RIPK3DRHIM-1xFV or RIPK3-1xFV following dimerizer treatment. Consistent with our hypothesis, we found RIPK3ΔRHIM-1xFV predominantly in a gel-shifted complex that is consistent with dimer formation, whereas RIPK3-1xFV was present in a combination of dimers and larger oligomeric complexes (Figure 1e). We therefore sought to use our constructs to define the regulation of RIPK3 oligomerization and activation.

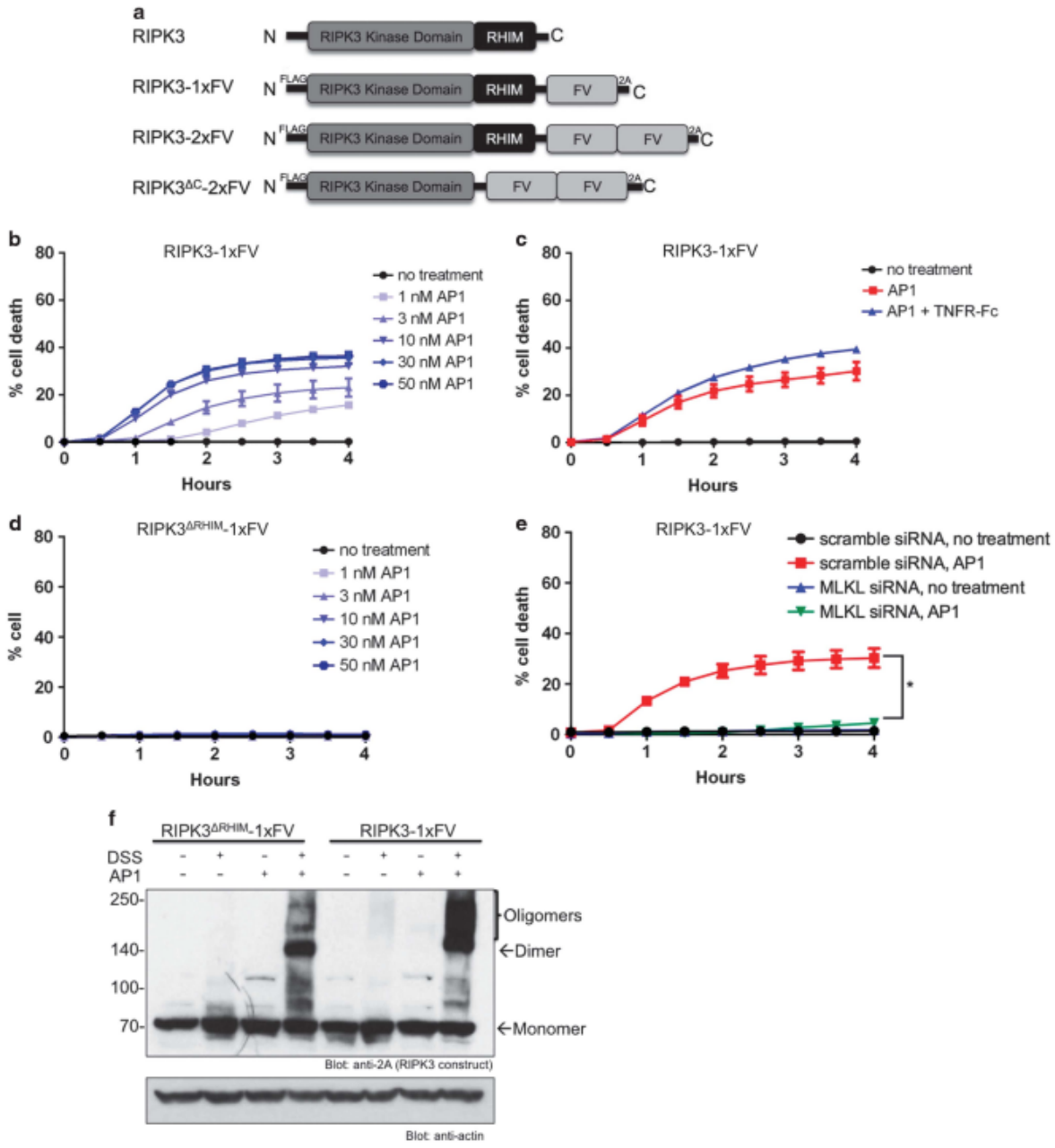


Figure 3.1. RIPK3 dimerization seeds a RHIM-dependent necrosome complex. (a) Schematic representation of the dimerizable and oligomerizable RIPK3 constructs used in this study. These constructs were cloned upstream of a T2A-GFP sequence, such that RIPK3 constructs contain both N-terminal FLAG and C-terminal 2A epitope tags. (b and c) NIH-3T3 cells stably expressing RIPK3-1xFV were treated with indicated concentrations of AP1 (b), or with 30 nM AP1 in the presence or absence of 200 ng/ml TNFR1-Fc (c), and cell death was assessed over time using an IncuCyte imaging system. (d) NIH-3T3 cells stably expressing RIPK3DRHIM-1xFV were treated with increasing doses of dimerizer. (e) NIH-3T3 cells stably expressing RIPK3-1xFV were transfected with indicated siRNAs. Seventy-two hours later cells were treated with 30 nM AP1 and cell death was assessed. *P ¼ 0.0001 (f) NIH-3T3 cells stably

expressing indicated constructs were treated as indicated, lysed and necrosome complexes were covalently cross-linked using DSS. Resulting complexes were resolved by western blotting. Nec1 and zVAD were used at 30 and 50 mM, respectively, throughout.

RHIM-dependent formation of RIPK3 complexes is controlled by RIPK1 and caspase-8

Current models of receptor-driven necroptosis involve the scaffolding and activation of RIPK1 at a plasma membrane receptor, followed by its translocation to the cytosol and the recruitment of both caspase-8 and RIPK3 into a 'necrosome' complex;[1] RIPK1 can activate RIPK3 in this complex,[5] while caspase-8 can suppress this activation.[25,26] We tested whether similar dynamics might influence the receptor independent formation and propagation of RIPK3 complexes triggered by RIPK3 dimerization. To our surprise, we found that addition of the caspase inhibitor zVAD (Figure 2a), or siRNA-mediated knockdown of caspase-8 (Supplementary Figures S2A and B) notably increased the rate and magnitude of necroptosis triggered by RIPK3-1xFV dimerization, in a manner analogous to that observed with TNF-driven RIPK3 activation in these cells (Supplementary Figure S1B). However, the effects we observed were independent of TNF receptor engagement, as the increased death observed on treatment with AP1 and zVAD were unaffected by the TNF-blocking reagent TNFR1-Fc (Supplementary Figure S2B). Conversely, treatment of cells expressing RIPK3-1xFV with the RIPK1 inhibitor necrostatin-1 (Nec1),3 which blocks TNF-induced necroptosis in these cells (S1C), suppressed RIPK3 dimerization-induced cell death (Figure 2c). These findings indicate that caspase-8 and RIPK1 may intrinsically regulate the initiation and propagation of RIPK3 oligomers in the cytosol, independent of TNF receptor-mediated signaling pathways. To more directly test this idea, we performed DSS crosslinking experiments to visualize the formation and stability of RIPK3 complexes following RIPK3 dimerization, when either caspase-8 or RIPK1 were inhibited (Figure 2d and Supplementary Figure S2C). Consistent with our hypothesis, we

found that dimerization of RIPK3 in the presence of zVAD led to an increased shift of RIPK3 into higher-molecular weight complexes consistent with RIPK3 oligomers, while RIPK3 dimerization in the presence of Nec1 diminished the appearance of these complexes. As a further test of these ideas, we immunoprecipitated RIPK3-1xFV or RIPK3DRHIM-1xFV from cells treated with AP1 in the presence of Nec1 or zVAD.

Previous work has shown that caspase inhibition can stabilize a caspase-8- and RIPK1-containing necrosome complex following TNF treatment,25,32 and we observed a similar phenomenon following RIPK3 dimerization (Figure 2e and Supplementary Figure S2E). Dimerization itself led to limited recruitment of RIPK1 and caspase-8 to the RIPK3 complex, while addition of zVAD increased association of these proteins. Inhibition of RIPK1 by Nec1, by contrast, eliminated the formation of stable RIPK1- and caspase-8-containing complexes. Similarly, mutation of the RHIM domain of RIPK3 prevented necrosome formation on RIPK3 dimerization. Notably, however, probing crosslinked RIPK3 oligomers with antibodies for RIPK1, caspase-8, or MLKL did not reveal significant association of these proteins with high-molecular weight RIPK3 complexes (Supplementary Figure S2D), consistent with a model in which RIPK3 is the primary component of these RHIM-dependent oligomers, with other proteins acting to control their formation. Together, these data show that RIPK3 dimerization, in the absence of receptor signaling, is sufficient to nucleate the formation of a RHIM-dependent necrosome. Further, necroptotic signaling from this complex is potentiated by the kinase activity of RIPK1 and inhibited by caspase-8 in a manner analogous to – but independent of – that observed during TNF-mediated necroptosis.

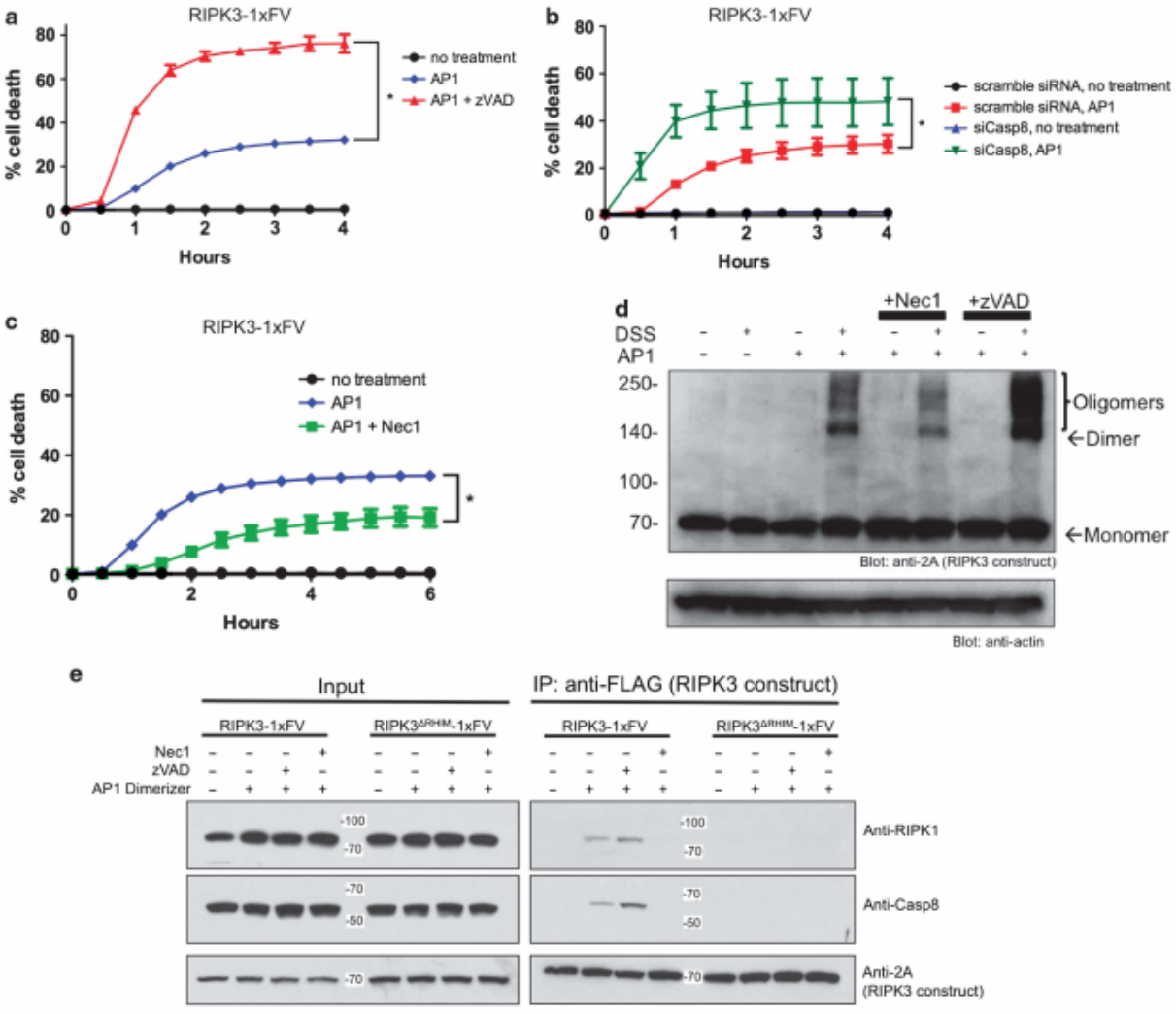


Figure 3.2. Receptor-independent RIPK3 oligomerization is regulated by RIPK1 and caspase-8. (a–c) NIH-3T3 cells stably expressing RIPK1-1xFV were treated with 30 nM AP1 and the indicated inhibitors, and cell death was assayed by IncuCyte. In b, cells were transfected with indicated siRNAs, then treated with AP1 72 h later. a: *P<0.0001, b: *P = 0.0157, c: *P = 0.0006. (d) NIH-3T3 cells stably expressing RIPK3-1xFV were treated with 30 nM AP1, as well as Nec1 or zVAD as indicated, and resulting complexes were resolved by western blotting. (e) NIH-3T3 cells expressing the indicated constructs were treated as shown for 30 min, then lysed and subjected to immunoprecipitation using an antibody to the FLAG epitopes expressed on the RIPK3 constructs. Immune complexes were purified and resolved using TruBlot reagents to avoid aspecific signals from immunoglobulins, as described. Nec1 and zVAD were used at 30 and 50 μM, respectively, throughout.

Oligomerization of RIPK3 is both necessary and sufficient to trigger necroptosis.

Based on these data we hypothesized that RIPK3 oligomerization itself is key to its activation, and that RIPK1 and caspase-8 act by regulating the initiation and propagation of RIPK3 oligomers. Accordingly, we reasoned that chemically induced oligomerization (rather than dimerization) of RIPK3 should eliminate the ability of RIPK1 and caspase-8 to control this process. To achieve this, we created constructs in which two FV domains are attached to the C-terminus of RIPK3, creating what we refer to as RIPK3-2xFV, with the goal of promoting AP1-induced crosslinking and oligomerization independent of the RHIM domain (Figure 1a). Notably, the application of AP1 to cells expressing RIPK3-2xFV resulted in a faster and more robust necroptotic response, to a degree comparable to that observed on dimerization of RIPK3-1xFV following caspase-8 inhibition or knockdown (Figure 3a compared with Figure 2a). Strikingly, although dimerization of a version of RIPK3 lacking a RHIM domain failed to induce any cell death response (Figure 1d), the addition of a second FV domain to create RIPK3ΔC-2xFV permitted robust necroptosis even in the absence of the RHIM domain (Figures 1a and 3b). As expected, cells expressing RHIM-less RIPK3ΔC-2xFV construct were non-responsive to TNF and zVAD (Supplementary Figure S1G.) Furthermore, addition of zVAD or Nec1 to cells expressing RIPK3-2xFV or RIPK3ΔC -2xFV did not alter magnitude or kinetics of cell death (Figures 3c and d). Consistent with a conclusion of chemically induced oligomerization, we observed large molecular weight RIPK3 complexes when cells expressing RIPK3-2xFV or RIPK3ΔC-2xFV were exposed to AP1 (Figure 3e). Interestingly, immunoprecipitation of RIPK3-2xFV following AP1 addition revealed interaction with RIPK1 and caspase-8, regardless of the presence of zVAD or Nec1 (Figure 3f). These data indicate that although RIPK1 and caspase-8 are recruited to the RIPK3 oligomers formed by this construct, enforced oligomerization of RIPK3 eliminates the ability of RIPK1 or caspase-8 to modulate RIPK3

activation. From this we can conclude that oligomerization of the RIPK3 kinase domain is both necessary and sufficient to trigger necroptosis, irrespective of the presence of the RHIM domain. Further, intrinsic control of this complex by caspase-8 and RIPK1 acts at, or upstream of, the formation of RIPK3 oligomers.

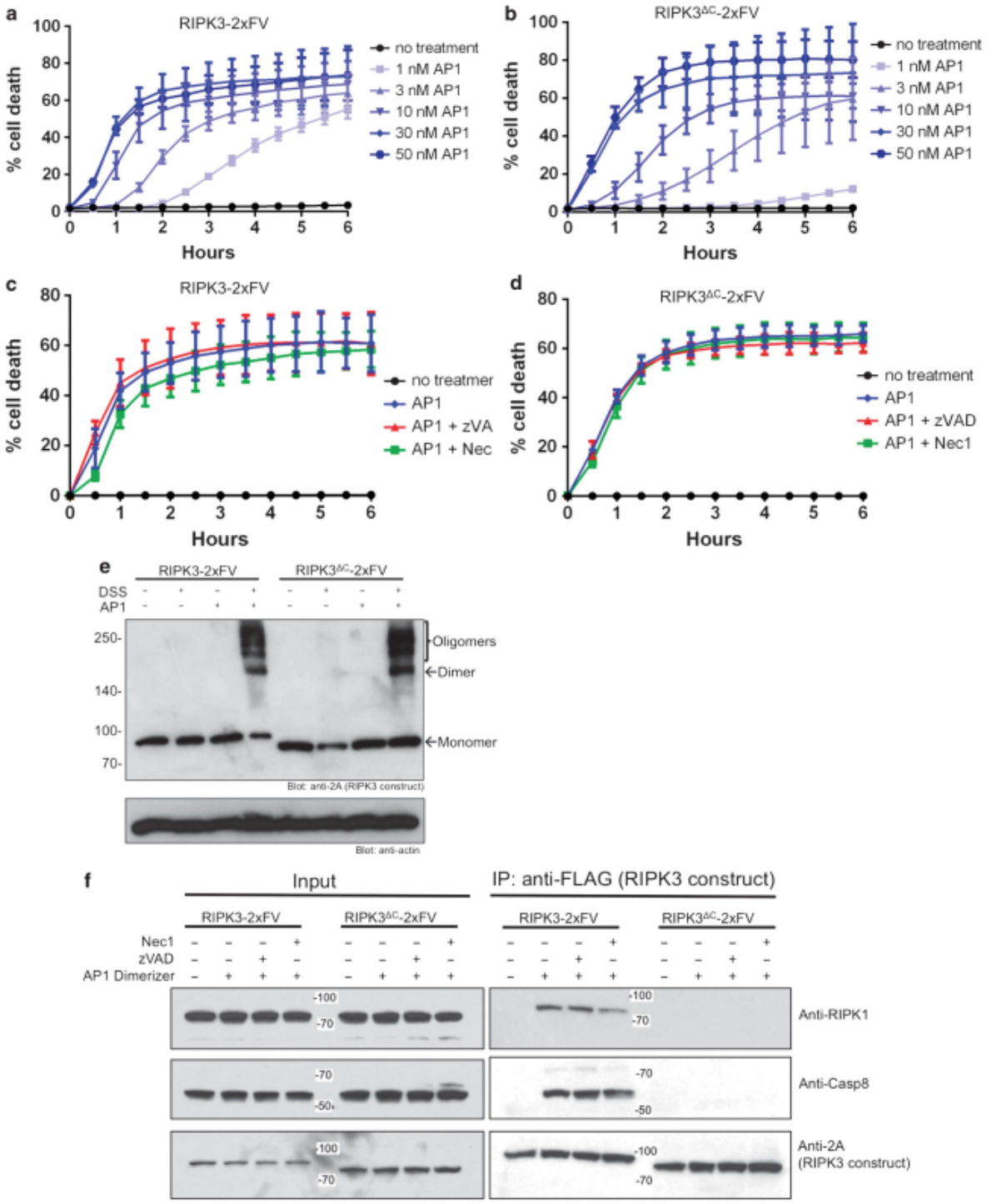


Figure 3.3. Chemically enforced RIPK3 oligomerization activates RIPK3 in the absence of the RHIM domain.

(a–d) NIH-3T3 cells expressing RIPK3-2xFV (a and c) or RIPK3DC -2xFV (b and d) were treated as indicated, and cell death was assessed using an IncuCyte as described. (e) NIH-3T3 cells stably expressing indicated constructs were treated as indicated, lysed and necrosome complexes were covalently cross-linked using DSS. Resulting complexes were resolved by

western blotting. (f) NIH-3T3 cells expressing the indicated constructs were treated as shown for 30 min, then lysed and subjected to immunoprecipitation using an antibody to the FLAG epitopes expressed on the RIPK3 constructs. Immune complexes were purified and resolved using TruBlot reagents to avoid aspecific signals from immunoglobulins, as described. Nec1 and zVAD were used at 30 and 50 mM, respectively, throughout.

RHIM-dependent RIPK3 oligomerization is inhibited by the presence of RIPK1 protein.

Our finding that 'seeding' of RHIM-dependent oligomers of RIPK3 via dimerization is inhibited by Nec1 (Figure 2c) implies that the kinase activity of RIPK1 drives the formation of RIPK3 oligomers; the recruitment of RIPK1 to RIPK3 dimers similarly supports the idea that RIPK1 may have a role in promoting the propagation of RIPK3 oligomers. To verify that our observations are not due to off-target effects of Nec1, we sought to confirm this finding by knocking down RIPK1 using siRNA (Supplementary Figure S2A). Unexpectedly, we found that although inhibition of RIPK1 with Nec1 blocked RIPK3 activation, RIPK1 knockdown significantly enhanced it (Figure 4a). This finding implies that the presence of the RIPK1 protein inhibits receptor-independent oligomerization of RIPK3, and that the inhibitory effects of Nec1 may rely on the scaffolding function of the chemically inhibited RIPK1 protein. Consistent with this idea, and with an on-target effect of Nec1, we found that the inhibitory effects of Nec1 on RIPK3 oligomerization were abrogated on knockdown of RIPK1 (Figure 4a compared with Figure 2c). These data imply that although the kinase activity of RIPK1 can potentiate RIPK3 oligomerization, RIPK1 is also required to exert intrinsic control of RIPK3 activation in the cytosol. We therefore reasoned that cells lacking RIPK1 should display reduced sensitivity to TNF-induced RIPK3 activation, but increased sensitivity to spontaneous activation of RIPK3. To directly test this idea, we fused RIPK3 to a destabilization domain (DD),33 creating a version of RIPK3 that is rapidly and constitutively degraded, but that accumulates in response to the DD-

binding drug, referred to as Shield (Supplementary Figure S3A). We confirmed that the DD-RIPK3 fusion protein accumulated in response to Shield administration (Figure 4b), and that Shield pre-treatment increased the sensitivity of NIH-3T3 cells expressing this construct to TNF-induced necroptosis (Supplementary Figure S3B). Furthermore, RIPK3 accumulation was also sufficient to trigger spontaneous necrosome formation and limited cell death in the absence of exogenous TNF. Consistent with RIPK3 accumulation triggering spontaneous necrosome formation, this cell death was unaffected by the TNF-blocking reagent TNFR1-Fc (Supplementary Figure S3C), and could be abrogated by the K51A insertional mutation of the active site of DD-RIPK3 (Figure 4b and Supplementary Figure S3D). We next used this system to evaluate RIPK1 as an intrinsic inhibitor of RIPK3 activation in the absence of receptor signaling. Consistent with canonical roles of caspase-8 and RIPK1 following TNFR1 ligation, knockdown of caspase-8 greatly sensitized cells expressing low levels of RIPK3 to TNF-induced cell death, while knockdown of RIPK1 did not (Figure 4c). However, when either caspase-8 or RIPK1 expression were silenced in the presence of Shield drug, the stabilization of RIPK3 was sufficient to support spontaneous, TNF-independent activation of DD-RIPK3 (Figure 4d). Importantly, addition of the RIPK1 inhibitor Nec1 to DD-RIPK3-expressing cells decreased cell death triggered by RIPK3 accumulation, and this effect was eliminated by RIPK1 siRNA-mediated knockdown, demonstrating that the effects of Nec1 are on-target and depend on the presence of the RIPK1 protein (Figure 4e). To further explore these findings, we stably reconstituted murine embryonic fibroblast cells lacking both RIPK1 and RIPK3 (RIPK1/3 DKO mouse embryonic fibroblast (MEF)) with RIPK1 or a catalytically inactive RIPK1K45A mutant protein, and also expressed DD-RIPK3 in these cells (Figure 5a). Consistent with a role for RIPK1 in preventing RIPK3 activation at steady state, we were unable to achieve stable

expression of DD-RIPK3 in RIPK1/3 DKO MEF cells not reconstituted with RIPK1 (not shown). Moreover, consistently we found that RIPK3 accumulation triggered spontaneous necroptosis to a notably greater degree in DKO MEF cells reconstituted with RIPK1, as compared with those expressing RIPK1K45A (Figure 5b). Nec1 inhibited RIPK3 activation and necroptosis in cells expressing RIPK1, but had no effect on cells expressing RIPK1K45A. These data indicate that although RIPK1 can drive receptor-induced RIPK3 activation and necroptosis, it also acts as an intrinsic suppressor of RIPK3 necrosome formation in the absence of receptor signaling. Furthermore, the RIPK1 inhibitor Nec1 potentiates this inhibitory function by creating an inactive form of RIPK1, an effect that is recapitulated by a catalytically inactive form of RIPK1. Genetic elimination and chemical inhibition of RIPK1 thereby have opposing effects.

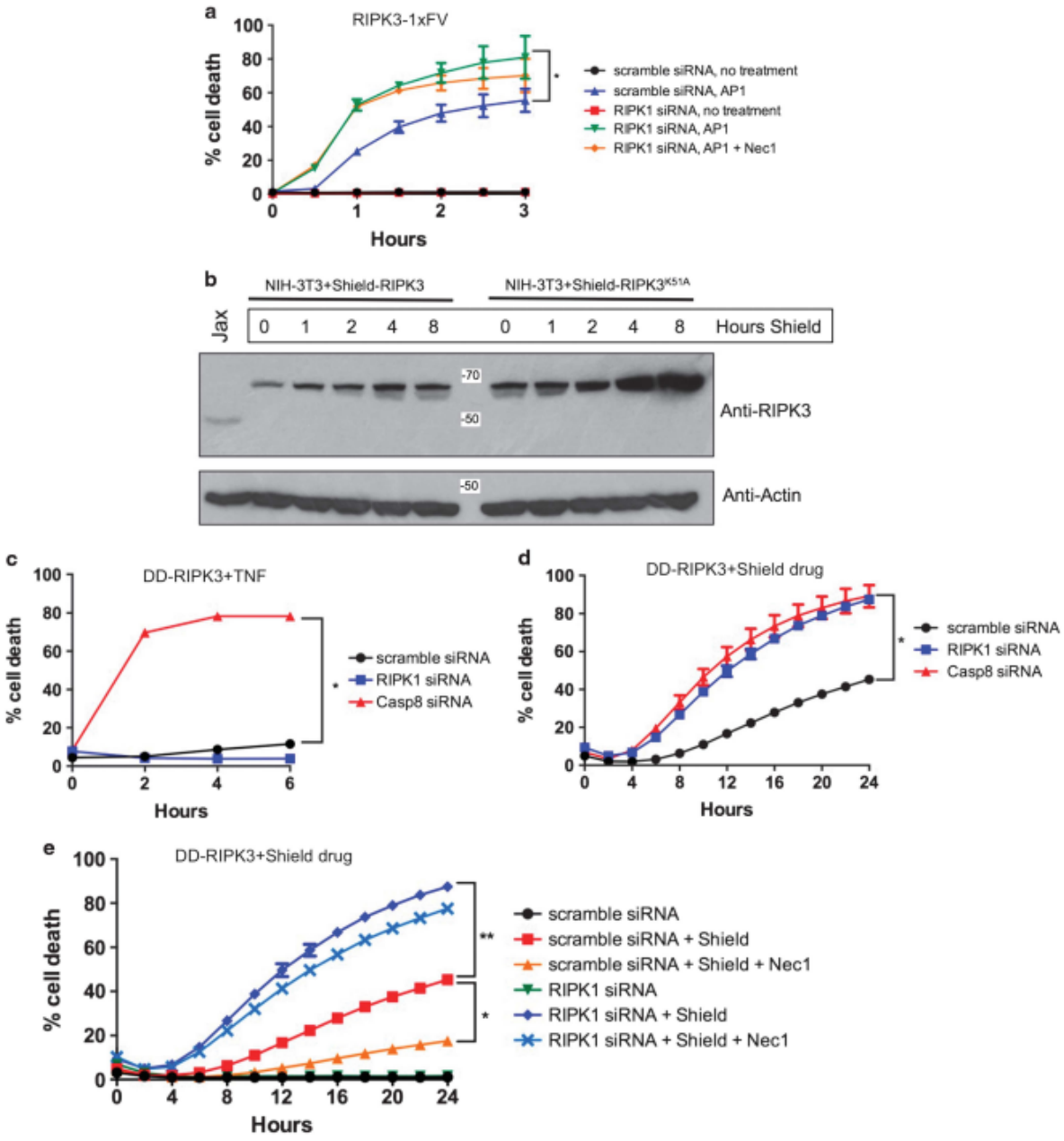


Figure 3.4. The presence of RIPK1 inhibits spontaneous RIPK3 oligomerization and necroptosis. (a) NIH-3T3 cells expressing RIPK3-1xFV were transfected with indicated siRNAs, then with 30 nM AP1 or 30 mM Nec1 72 h later. For scramble versus RIPK1 siRNA treated with AP1, *P = 0.0024. (b) NIH-3T3 cells stably expressing DD-RIPK3 or DD-RIPK3^{K51A} were treated with 1 mM Shield drug for indicated times, then lysed and resolved by western blotting. Jackson immortalized fibroblasts (Jax) expressing endogenous RIPK3 are included as a control. NIH-3T3 cells expressing DD-RIPK3 were transfected with indicated siRNAs, then treated 72 h later with 1 ng/ml recombinant TNF (c) or 1 mM Shield drug (d and e) and 30 mM Nec1 as indicated. c: *P<0.0001, d: *P<0.0001, e: *P=0.0001, **P<0.0001

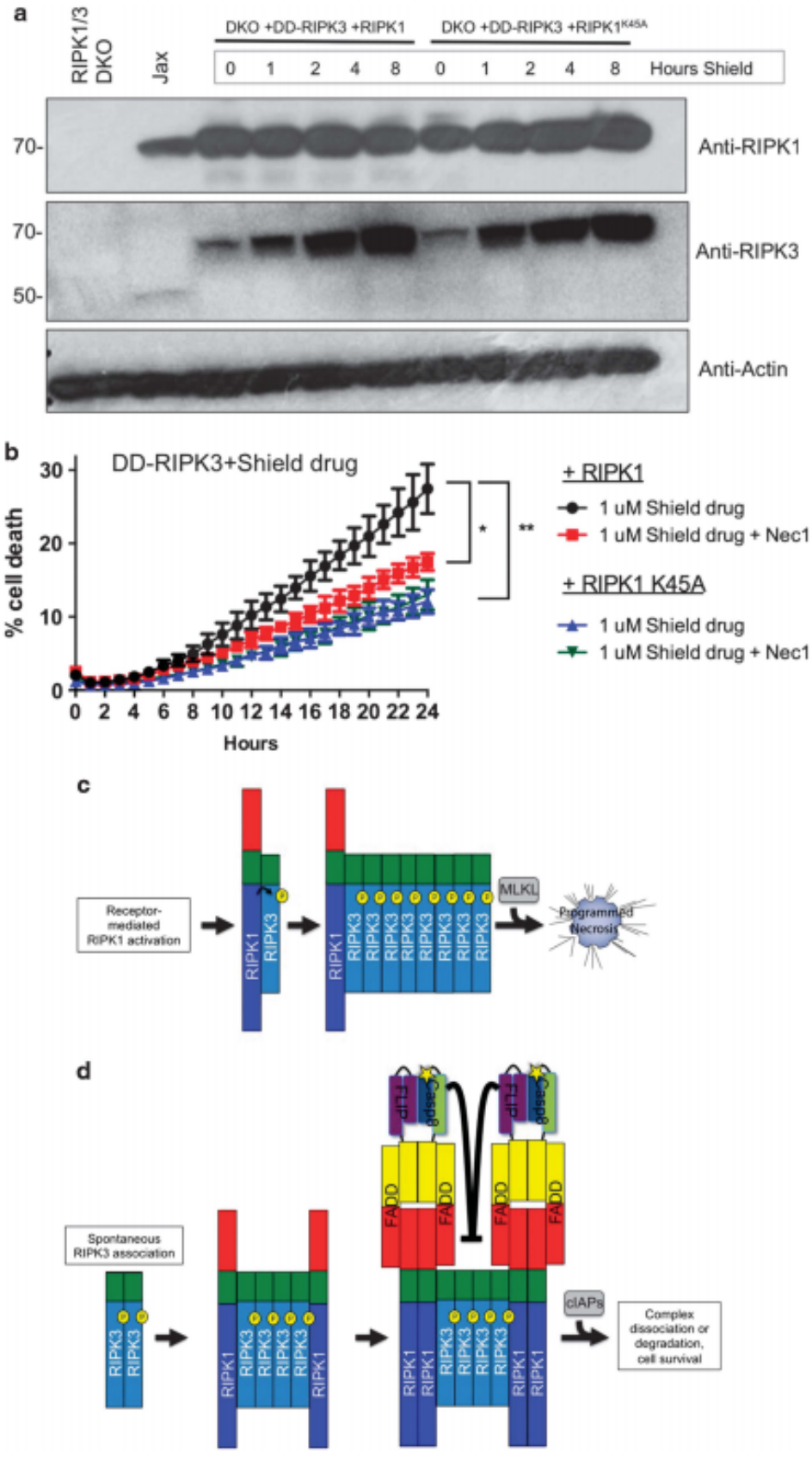

a
RIPK1/3 DKO
Jax
DKO +DD-RIPK3 +RIPK1
DKO +DD-RIPK3 +RIPK1K45A
0 1 2 4 8 0 1 2 4 8 Hours Shield
70-
Anti-RIPK1
70-
50-
Anti-RIPK3
Anti-Actin
b
DD-RIPK3+Shield drug
% cell death
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Hours
*
**
+ RIPK1
1 uM Shield drug
1 uM Shield drug + Nec1
+ RIPK1 K45A
1 uM Shield drug
1 uM Shield drug + Nec1
c
Receptor-mediated RIPK1 activation
RIPK1
RIPK3
MLKL
Programmed Necrosis
d
Spontaneous RIPK3 association
FADD
cIAPs
Complex dissociation or degradation, cell survival

Figure 3.5. Catalytically inactive RIPK1 mimics the suppressive effects of Nec-1 on spontaneous RIPK3 oligomerization and necroptosis.
(a) RIPK1/RIPK3 DKO MEF cells were stably transduced with wild-type murine RIPK1 or RIPK1K45A, then stably transduced with DD-RIPK3. These cell lines were treated with Shield drug as indicated, then lysed and resolved by western blotting. Jax cells are included as a control for endogenous RIPK expression. (b) The cell lines depicted in a were treated with Shield drug in combination with Nec-1 as indicated. *P ¼ 0.0154, **P ¼ 0.0027. (c and d) A speculative model for the control of RIPK3 activation by RIPK1; RHIM domains are depicted in green, death domains in red and death effector domains in yellow. (c) When RIPK1 is activated by receptor signals, it binds to and phosphorylates RIPK3 via RHIM–RHIM interactions. This leads to RHIM-driven RIPK3 oligomerization, MLKL recruitment and necroptosis. (d) In the absence of receptor signals, spontaneous RIPK3 activation recruits RIPK1, which in turn recruits caspase-8/FLIP complexes. This leads to inhibition of RIPK3 oligomerization, possibly via cIAP-mediated degradation of the nascent RIPK3 complex.

3.4 DISCUSSION

In this study, we use inducible protein interaction systems to explore the mechanisms of RIPK3 activation. Our findings indicate that the formation of a RIPK3 dimer via a C-terminal dimerization domain, while not itself sufficient to activate RIPK3, is able to ‘seed’ an RHIM-dependent complex whose propagation leads to RIPK3 activation. Interestingly, we find that the stability of this complex, and by extension the activation of RIPK3 and necroptosis, is controlled by caspase-8 and RIPK1. Both caspase-8 and RIPK1 are recruited to the RIPK3 oligomer, and inhibition of caspase-8 potentiates RIPK3 oligomerization and necroptosis, while inhibition of RIPK1 inhibits these processes. Surprisingly, however, although siRNA-mediated knockdown of caspase-8 has a similar effect on its chemical inhibition, knockdown and inhibition of RIPK1 had opposing effects on RIPK3 oligomerization and activation. Although RIPK1 inhibition reduced RIPK3 activation, siRNA-mediated knockdown of RIPK1 notably potentiated it. From this, we can conclude that although the kinase activity of RIPK1 can contribute to RIPK3 activation, recruitment of RIPK1 is not required for – and indeed inhibits – the formation of RIPK3

oligomers. Consistent with this model, a recent study demonstrated that a knock-in mouse expressing catalytically inactive RIPK1D138N is viable, unlike RIPK1 knockout animals which die perinatally.34 This raises the intriguing possibility that the perinatal lethality of RIPK1-deficient animals may be the result of unchecked RIPK3 activity, possibly independently of receptor signaling. In a companion to our study, Han and colleagues35 used similar induced-interaction systems to define the minimal complex necessary for necroptotic signaling. Although their results are largely compatible with ours, one key difference exists: they find that enforced RIPK3 dimerization, even in the absence of a RHIM domain, is sufficient to trigger RIPK3 autophosphorylation, MLKL activation and necroptosis. A likely explanation for this apparent discrepancy is that Han and colleagues appended dimerization domains adjacent the N-terminal kinase domain of RIPK3, while our study used C-terminal dimerization domains in an effort to mimic the action of the C-terminal RHIM domain. It is therefore likely that while forcing dimerization via the N terminus of RIPK3 leads to proximity-induced autophosphorylation and MLKL binding, a lack of structural constraint between the N-terminal kinase domain and the C-terminal RHIM means that C-terminal dimerization – as would occur with RHIM-RHIM interactions – does not. These data point to a model in which RIPK3 dimerization leads to the exposure of the RHIM domain, allowing the recruitment of both RIPK1 and additional molecules of RIPK3 into the amyloid-like oligomer previously described.17 When RIPK1 is present in this structure, it mediates the recruitment of caspase-8 which, in concert with its paralog cFLIPL, promotes destabilization and inhibition of the growing necrosome via interactions between the C-terminal death domain of RIPK1 and the caspase-8 adapter Fas-associated protein with a death domain (FADD).25 Nec1 treatment promotes this inhibition by effectively creating a catalytically inactive form of RIPK1, which nonetheless is able to act as a molecular scaffold to

recruit inhibitory caspase-8. The precise cleavage and phosphorylation events that govern necrosome assembly and stability remain poorly understood,34 but degradation of nascent RIPK3 complexes by the cIAPs has been shown to play a key role in the suppression of RIPK3 activation.18,19,36 We were unable to recover stable RIPK1/RIPK3 complexes following RIPK3 dimerization in the presence of Nec1, consistent with degradation of these complexes. Notably, however, we found that chemically enforced oligomerization potentiated RIPK3 activation and eliminated the ability of caspase-8 and RIPK1 to control this process, despite their recruitment to RIPK3 oligomers. Together, these findings are consistent with the idea that RIPK1-dependent recruitment of caspase-8 to nascent RIPK3 complexes targets them for degradation before RIPK3 oligomer formation. RIPK3 oligomerization may thus represent a 'point of no return' for necroptotic signaling (Figures 5c and d). Although many of our findings were generated using inducible protein interaction systems, they have clear implications for RIPK3 signaling under physiological conditions. Consistent with structural studies of the RHIM domains17 as well as other amyloid-forming proteins, it is likely that the RHIM domains of RIPK3 proteins in the cytosol of healthy cells are somewhat 'sticky,' undergoing limited interactions in the absence of exogenous signals. Because of the self-propagating nature of these structures, it is important that cells have in place mechanisms to limit these interactions in the absence of pro-death signaling. Recruitment of the RIPK1 RHIM domain to RIPK3 oligomers, in the absence of other signals, may thereby recruit suppressive proteins to limit oligomer propagation. Understanding how RIPK1 is activated at the molecular level – that is, how recruitment to the TNFR1 complex and subsequent posttranslational modification potentiates the kinase activity of RIPK1 – will be important in understanding how RIPK1 makes the switch from inhibitor to activator of RIPK3 oligomerization during receptor signaling.

3.5 MATERIALS AND METHODS

Constructs and cell lines

RIPK3-FV chimeric proteins were constructed by cloning full-length murine RIPK3, catalytically dead RIPK3^{K51A}, RIPK3 lacking the final 42 amino acids (RIPK3$^{\Delta C}$), or RIPK3 bearing a 4 amino acid substitution in the RHIM motif, VQIG→AAAA, (RIPK3$^{\Delta RHIM\Delta}$) upstream of either one or two copies of FKBP carrying the F36V mutation, herein called "FV" domains. When two FV domains were used, the first copy contained silent mutations to prevent DNA recombination. These RIPK3-FV fusion proteins were cloned into pBabe-Puro retroviral vectors containing T2A ribosome-skipping sequences derived from porcine teschovirus-1 (EGRGSLLTCGDVEENPGP) upstream of eGFP, creating bicistronic constructs in which RIPK3-FV and GFP are translated from the same mRNA, but separate upon translation to generate distinct proteins. FLAG tags were added to the N-terminus of RIPK3 during this cloning step. The resulting constructs therefore take the general form FLAG-RIPK3-FV-T2A-GFP in pBabe-Puro vectors, and the expressed RIPK3 fusion proteins, once separated from GFP, contain both an N-terminal FLAG and a C-terminal 2A epitope.

These constructs were transduced into NIH-3T3 cells using standard protocols for helper-dependent retroviral transduction. Transduced cells were selected for 5 days in 1μg/ml Puromycin, then grown to confluence and sorted twice for homogenous GFP expression. A minimum of two distinct, separately-derived stable cell lines expressing each of these proteins was generated, and experiments presented are representative of results obtained with both cell lines. All cell lines were maintained in D-MEM (Fisher, SH30022FS) supplemented with 10%

FCS (Sigma, 0926-500ML), 29.2g/L glutamine (Fisher, SH3003402), 10,000U/mL penicillin and 10,000µg/mL streptomycin (Fisher, SV30010), and grown at 37 degrees in 5% CO_2.

Chimeric proteins composed of RIPK3 fused to the destabilization domain (DD, described in Ref. 31) were created via recombinant PCR, to produce a fragment composed of an N-terminal FLAG tag followed by the destabilization domain, then full-length murine RIPK3 or $RIPK3^{K51A}$. These constructs were cloned into the pRRL lentiviral vector downstream of an MND promoter, and upstream of a T2A-GFP-T2A-Puromycin resistance cassette. Thus, cells transduced with these vectors produce FLAG-DD-RIPK3, GFP, and the puromycin resistance marker as separate proteins from a single mRNA, and both DD-RIPK3 and GFP carry a C-terminal 2A epitope. These constructs were expressed in NIH-3T3 cells via standard helper-dependent lentiviral transduction, and resulting cells were selected in 2µg/ml puromycin. Stable expression was confirmed by flow cytometric analysis.

"Jax" cells are a line of SV40 immortalized C57Bl/6 murine embryonic fibroblasts produced by the Jackson Laboratory. These cells express endogenous RIPK3 and undergo rapid necroptosis in response to TNF+zVAD treatment. These cells were a kind gift of Dr. Dan Stetson.

Cell Death assays

Cell death assays were carried out using a 2-color IncuCyte Zoom in-incubator imaging system (Essen Biosciences.) Briefly, this system allows fully automated imaging of cells at set intervals in phase contrast as well as both red and green fluorescent channels. Cell death assays were carried out by treating cells with death-inducing compounds in 24 well tissue culture vessels (100,000 cells/well), in the presence of 100nM of the cell-impermeable DNA binding

fluorescent dyes Sytox Green (Life Technologies S7020) or Yoyo-3 (Y3606), which are excluded from healthy cells but rapidly enter dying cells upon membrane permeabilization, in a manner analogous to propidium iodide. Resulting images were analyzed using the software package supplied with the IncuCyte imager, which allows precise analysis of the number of Sytox Green or Yoyo-3 positive cells present in each image. An example of cells undergoing necroptosis and the results of dead cell quantification are presented in Supplemental Movies 1 and 2. For each experiment, a minimum of three separate wells were treated with each experimental condition, and a minimum of 4 image fields were assessed per well. Percent cell death was calculated by treating a minimum of three distinct wells in each experiment with 100nM of the cell permeable fluorescent dye Syto Green (S7559), which allows quantification of the total number of cells present in each field. Dead cell events acquired via Sytox Green or Yoyo-3 staining were divided by this total cell number to yield percent cell death at each timepoint. Error bars represent standard deviation from the mean of a minimum of three independent wells. Each result depicted is representative of at least 4 distinct experiments, each of which contained at least 3 technical replicates.

Chemicals, compounds, and siRNAs.

The following compounds were used: Recombinant murine TNF (Peprotech 315-01A) was used at 1ng/ml unless otherwise specified; AP1 (Now commercialized by Clontech as "B/B Homodimerizer," catalog #635059) was dissolved in ethanol to a concentration of 100μM, then diluted in culture media to a final concentration of 30nM unless otherwise indicated. zVAD (SM Biochemicals SMFMK001) was dissolved in DMSO to a concentration of 50mM, then diluted to 50μM in culture media; Necrostatin-1(Sigma N9037-10MG) was dissolved in DMSO

to a concentration of 30mM, then diluted to 30μM in culture media; TNFR-Fc (Fisher 430-RI-050) was used at a final concentration of 200ng/ml; Shield drug was a kind gift of Dr. Tom Wandless and was used at a final concentration of 1μM.

SiGenome SMARTpool siRNAs were purchased from Dharmacon/Fisher. Pools targeting murine MLKL(M-061420-01), murine RIPK1 (M-040150-01), and murine caspase-8 (M-043044-01), as well as a non-targeting "scramble" pool (D-001206-14), were used. Two microliters of a 50μM stock of these siRNAs were delivered to cells in 6-well format using Lipofectamine siRNA-Max reagent (Life Technologies 13778150) according to the manufacturer's instructions. Forty-eight hours later, these cells were re-plated into 24-well format for cell death assays, or harvested for western blot analysis of knockdowns.

Antibodies and Immunoprecipitation

The following antibodies were used: Anti-caspase-8 (Enzo, 1G12, ALX-804-447-C100), anti-RIPK1 (BD 610458), anti-RIPK3 (Imgenex IMG-5523-2), rabbit anti-FLAG (Abcam AB1162), anti-actin (Millipore MAB1501). The anti-2A antibody was a kind gift from Dr. Dario Vignali. The anti-MLKL antibody was a kind gift from Dr. Warren Alexander[32]. Secondary antibodies were purchased from Santa Cruz Biotechnology (mouse sc-2005 , rat sc-2006, rabbit sc-2313). These antibodies were used for western blotting of proteins separated using SDS-PAGE pre-cast gels (Invitrogen) and transferred to PVDF membranes. Membranes were incubated with primary and HRP-conjucated secondary antibodies in TBS-T buffer containing 5% non-fat milk, then detected using ECL reagents (Pierce). Detection was accomplished using either standard autoradiography film (Pierce) or an Chemidock electronic luminescence detection platform (ChemiDoc™ XRS+ System, # 170-8265).

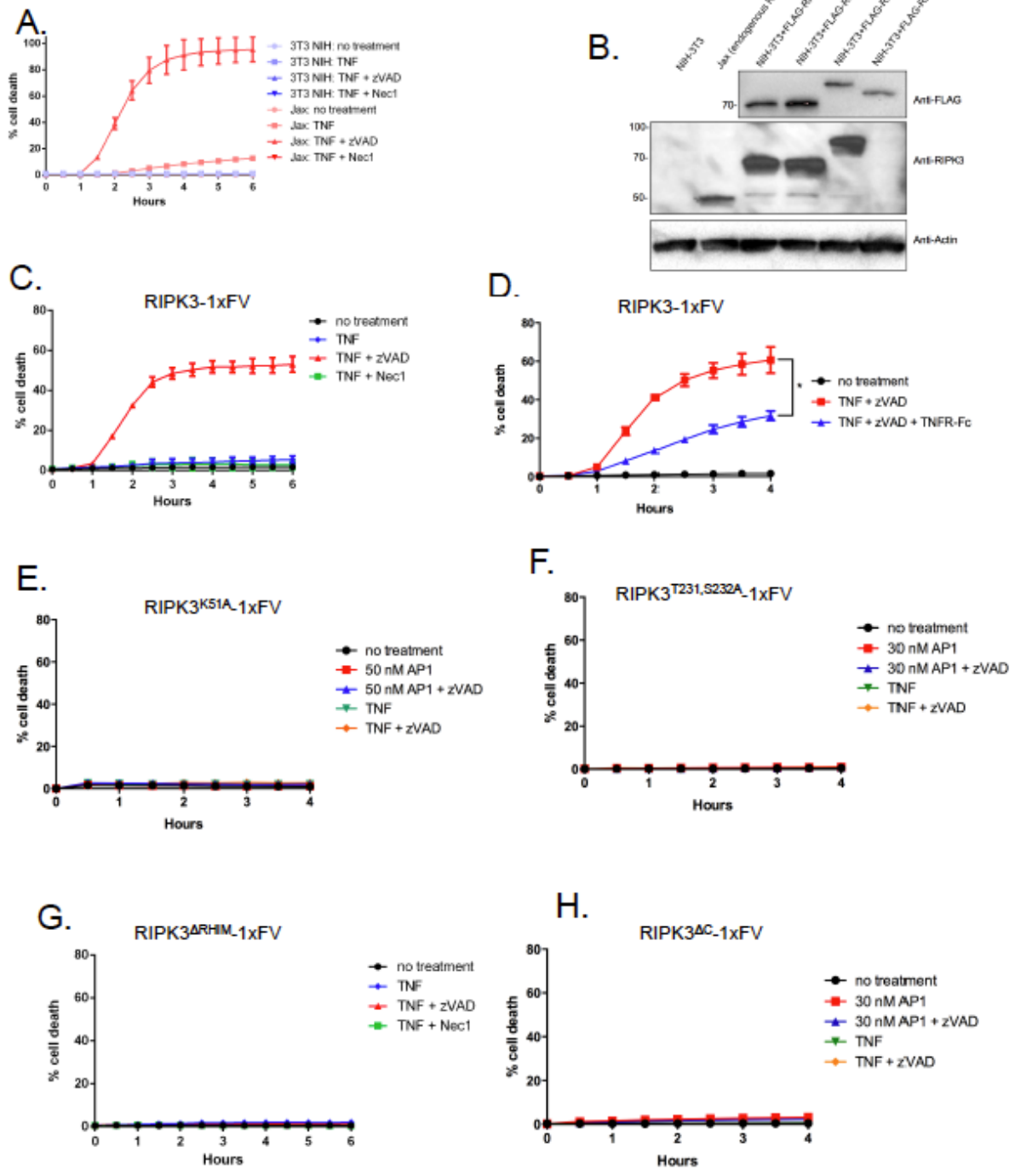
Immunoprecipitation of necrosome complexes were carried out using rabbit anti-FLAG antibodies, and TruBlot IP reagents, now sold by Rockland Immunochemicals, to eliminate aspecific signals from immunoglobulin heavy and light chains. Briefly, cell lines were treated as indicated and lysed in NP40 buffer (30mM Tris, 150mM NaCl, and 1% NP40) supplemented with protease inhibitors. Twenty micrograms of each sample were reserved for "input," wile 500μg of total protein from each sample were immunoprecipitated with 0.5μg antibody conjugated to 30μL TruBlot anti-Rabbit IgG beads. Immunocomplexes were washed 4 times in NP40 buffer, eluted by boiling, then run on western blot. Western blots were analyzed using standard primary antibodies, but TruBlot anti-Rabbit and anti-Mouse HRP-conjugated secondary antibodies were used. A standard anti-Rat-HRP secondary was used to detect caspase-8.

DSS Crosslinking

A confluent monolayer of the indicated cells was incubated with the appropriate treatment for 30 minutes at 37 degrees. Cells were lysed in a modified, Tris-free NP40 buffer (30mM HEPES pH 7.4, 150 mM NaCl, and 1% NP40) without protease inhibitors. Protein lysate was quantified according to standard BCA assay (Pierce) and fifty micrograms of each sample was treated with 0.1 mM DSS crosslinking agent (Thermo Scientific, #21658) for 30 minutes at room temperature; the reaction was quenched by the addition of Tris-HCl pH 7.2 to a final concentration of 50mM for 15 minutes. Crosslinked samples were analyzed by Western blot as described above, using the anti-2A primary antibody for detection.

3.1 SUPPLEMENTARY INFORMATION

Orozco et al. Supplementary Fig.1



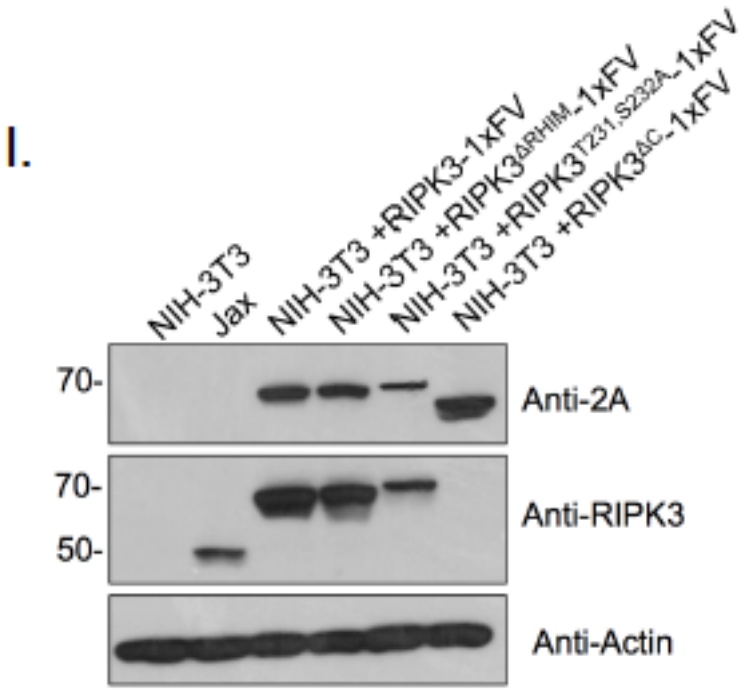


Figure 3.6. Orozco *et al.* CDD 2014, Supplementary Figure 1.
A) Jax cells, which express endogenous RIPK3, or NIH-3T3 cells, which do not, were treated with 10ng/ml recombinant TNF along with inhibitors as indicated. B) Lysates from Jax cells, or NIH-3T3 cells stably expressing indicated constructs, were resolved by Western blot using the indicated antibodies. Note that the RIPK3 antibody used recognizes an epitope in the C-terminus, which is lacking in the RIPK3ΔC construct. C&D) NIH-3T3 cells stably expressing RIPK3-1xFV were treated with recombinant TNF, 30μM Nec1, 50μM zVAD, or 200ng/ml TNFR1-Fc as indicated. *p =0.0002. E-H) NIH-3T3 cells stably expressing catalytically inactive RIPK3K51A-1xFV, phosphorylation site mutant RIPK3T231A,S232A-1xFV, RHIM domain point mutant RIPK3ΔRHIM-1xFV, or RHIM-truncated RIPK3ΔC-1xFV were treated as indicated. I) NIH-3T3 cells stably expressing the indicated constructs were lysed and resolved by western blotting. Jax cells are included as a control for endogenous RIPK3 expression.

Orozco et al, Supplementary Fig. 2

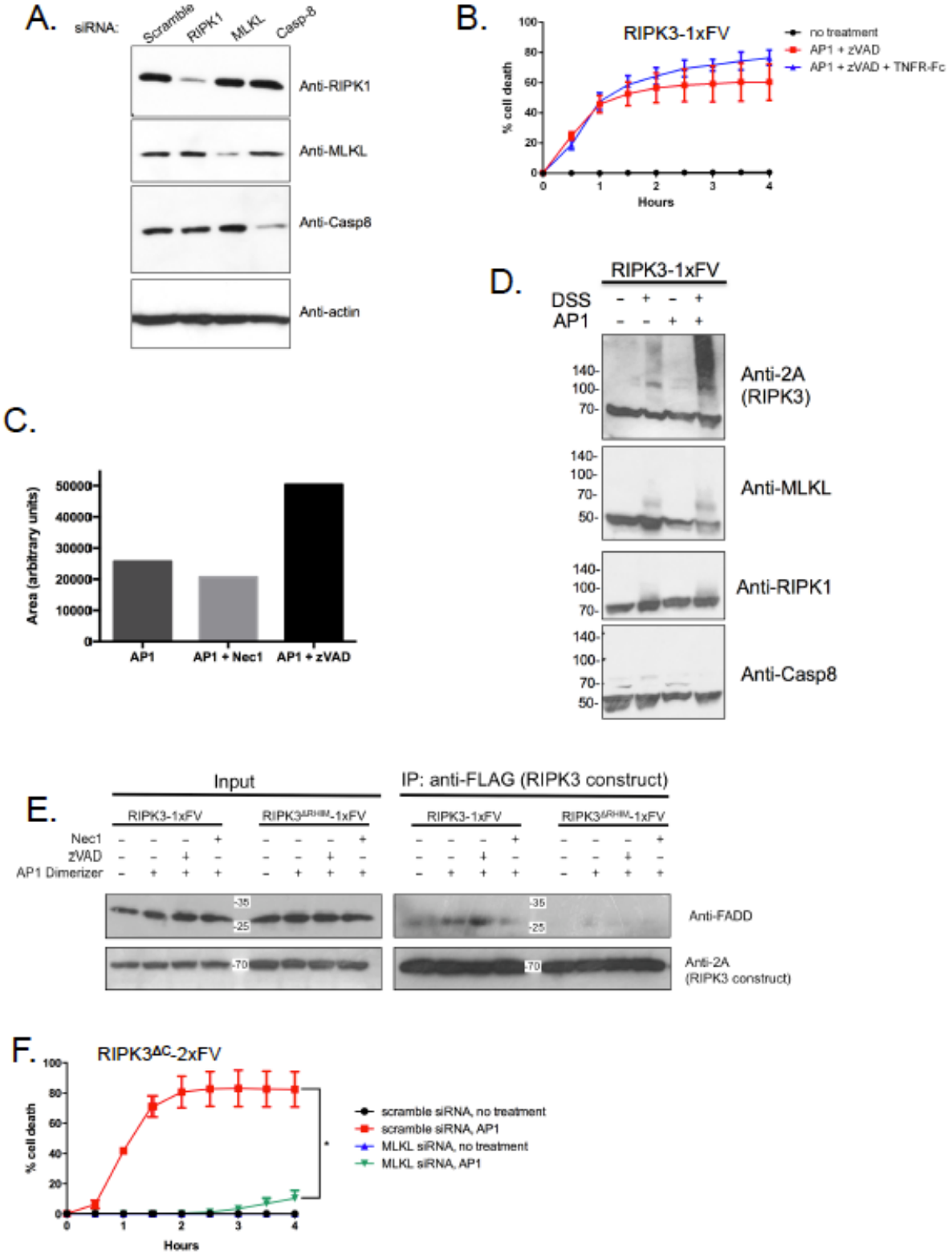
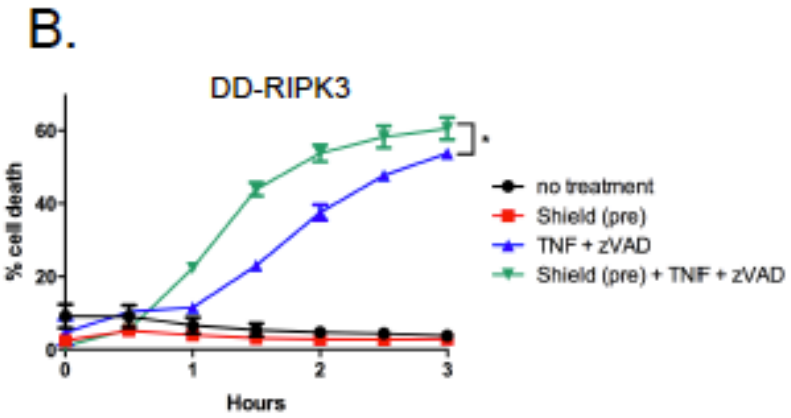
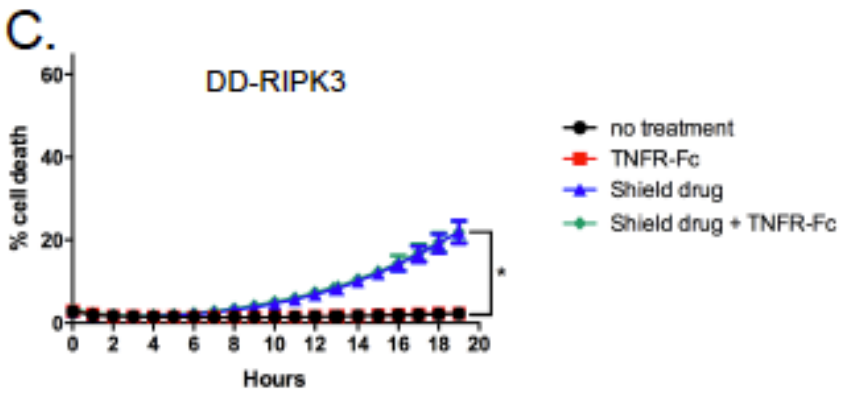


Figure 3.7. Orozco *et al.* CDD 2014, Supplementary Figure 2.
A) NIH-3T3 cells were transfected with indicated siRNAs. Seventy-two hours later, lysates were collected and expression of indicated proteins was assessed by western blot. B) NIH-3T3 cells stably expressing RIPK3-1xFV were treated with 30nM AP1, 50μM zVAD and 200ng/ml TNFR1-Fc as indicated. C) Densitometric analysis of the RIPK3 dimer and oligomer bands depicted in Fig. 2D. D) 3T3-NIH cells stably expressing RIPK3-1xFV were treated with AP1, then lysates were collected and subjected to DSS-mediated chemical crosslinking. These complexes were then resolved by western blotting using the indicated antibodies. E) RIPK1-associated immunocomplexes were purified as described in Fig. 1E, and co-precipitation of FADD was assessed by western blotting. F) NIH-3T3 cells stably expressing RIPK3ΔC-2xFV were transfected with indicates siRNAs. Seventy-two hours later these cells were treated as indicated. All cell death measurements were performed using an IncuCyte bioimager as described.

Orozco et al., Supplementary Fig. 3





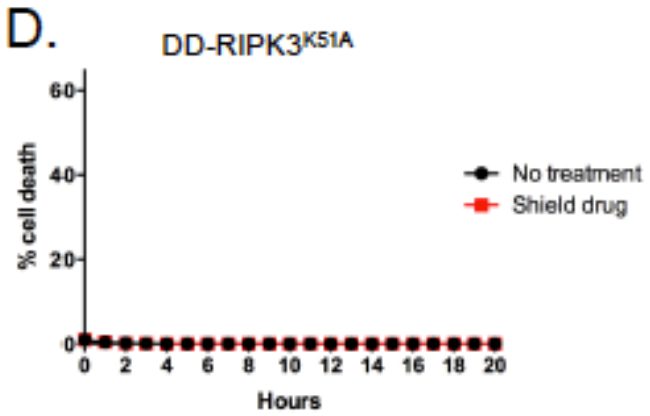


Figure 3.8. Orozco *et al.* CDD 2014, Supplementary Figure 3.
A) Schematic representation of the destabilization domain (DD)-RIPK3 construct used. A DD-RIPK3 chimeric open reading frame was created by recombinant PCR, then cloned upstream of a T2A-GFP-T2A-PURO sequence. Of note, both DDRIPK3 and GFP protein include a C-terminal 2A epitope. B) NIH-3T3 cells stably expressing DD-RIPK3 were pre-treated with 1μM Shield drug for 8 hours to stabilize RIPK3 expression, then treated with 1ng/ml TNF and 50μM zVAD as indicated. *P=0.0024 C) NIH-3T3 cells stably expressing DD-RIPK3 were treated with 1μM Shield drug and 200ng/ml TNFR-Fc as indicated. *p =0.0004 D) NIH-3T3 cells stably expressing catalytically inactive DD-RIPK3K51A were treated with 1μM Shield drug as indicated. All cell death measurements were performed using an IncuCyte bioimager as described.

3.2 Acknowledgements and funding

We thank Dr. Dan Stetson and Dr. Ram Savan, and their groups, for moral and material support. We also thank Dr. Stetson and Dr. David Rawlings for providing pRRL lentiviral constructs, Drs Dario Vignali and Warren Alexander for providing antibodies and Dr. Christopher Dillon for preparing RIPK1/3 DKO MEFs. We also thank Dr. Jiahuai Han for helpful discussions and input. This work was supported by NIH grant RAI108685A, Royalty Research Fund grant 65-0062 and UW Diabetes Research Center grant P30 DK017047, to AO. SO is supported by an NIH training grant 1T32AI106677-01 to the Department of Immunology of the University of Washington.

3.3 References

1. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 2010; 11: 700–714.

2. Vercammen D, Vandenabeele P, Beyaert R, Declercq W, Fiers W. Tumour necrosis factorinduced necrosis versus anti-Fas-induced apoptosis in L929 cells. Cytokine 1997; 9: 801–808.

3. Degterev A, Hitomi J, Germscheid M, Ch'en IL, Korkina O, Teng X et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 2008; 4: 313–321.

4. Lin Y, Choksi S, Shen HM, Yang QF, Hur GM, Kim YS et al. Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. J Biol Chem 2004; 279: 10822–10828.

5. Cho YS, Challa S, Moquin D, Genga R, Ray TD. Guildford M, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 2009; 137: 1112–1123.

6. Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 2009; 325: 332–336.

7. He S, Wang L, Miao L, Wang T, Du F, Zhao L et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 2009; 137: 1100–1111.

8. Vanden Berghe T, Vanlangenakker N, Parthoens E, Deckers W, Devos M, Festjens N et al. Necroptosis, necrosis and secondary necrosis converge on similar cellular disintegration features. Cell Death Differ 2010; 17: 922–930.

9. Thapa RJ, Nogusa S, Chen P, Maki JL, Lerro A, Andrake M et al. Interferon-induced RIP1/RIP3-mediated necrosis requires PKR and is licensed by FADD and caspases. Proc Natl Acad Sci USA 2013; 110: E3109–E3118.

10. He S, Liang Y, Shao F, Wang X. Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proc Natl Acad Sci USA 2011; 108: 20054–20059.

11. Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell Host Microbe 2012; 11: 290–297.

12. Upton JW, Kaiser WJ, Mocarski ES. Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe 2010; 7: 302–313.

13. Robinson N, McComb S, Mulligan R, Dudani R, Krishnan L, Sad S et al. Type I interferon induces necroptosis in macrophages during infection with Salmonella enterica serovar Typhimurium. Nat Immunol 2012; 13: 954–962.

14. Duprez L, Takahashi N, Van Hauwermeiren F, Vandendriessche B, Goossens V, Vanden Berghe T et al. RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity 2011; 35: 908–918.

15. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 1998; 8: 297–303.

16. Wang L, Du F, Wang X. TNF-alpha induces two distinct caspase-8 activation pathways. Cell 2008; 133: 693–703.

17. Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS et al. The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 2012; 150: 339–350.

18. Feoktistova M, Geserick P, Kellert B, Dimitrova DP, Langlais C, Hupe M et al. cIAPs block ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol Cell 2011; 43: 449–463.

19. Tenev T, Bianchi K, Darding M, Broemer M, Langlais C, Wallberg F et al. The ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol Cell 2011; 43: 432–448.

20. Chen W, Zhou Z, Li L, Zhong CQ, Zheng X, Wu X et al. Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling. J Biol Chem 2013; 288: 16247–16261.

21. Wu J, Huang Z, Ren J, Zhang Z, He P, Li Y et al. Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. Cell Res 2013; 23: 994–1006.

22. Sun L, Wang H, Wang Z, He S, Chen S, Liao D et al. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 2012; 148: 213–227.

23. Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J et al. Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci USA 2012; 109: 5322–5327.

24. Geserick P, Hupe M, Moulin M, Wong WW, Feoktistova M, Kellert B et al. Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. J Cell Biol 2009; 187: 1037–1054.

25. Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 2011; 471: 363–367.

26. Kaiser WJ, Upton JW, Long AB, Livingston-Rosanoff D, Daley-Bauer LP, Hakem R et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 2011; 471: 368–372.

27. Clackson T, Yang W, Rozamus LW, Hatada M, Amara JF, Rollins CT et al. Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity. Proc Natl Acad Sci USA 1998; 95: 10437–10442.

28. Oberst A, Pop C, Tremblay AG, Blais V, Denault JB, Salvesen GS et al. Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. J Biol Chem 2010; 285: 16632–16642.

29. Pop C, Oberst A, Drag M, Van Raam BJ, Riedl SJ, Green DR et al. FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. Biochem J 2011; 433: 447–457.

30. Chang DW, Yang X. Activation of procaspases by FK506 binding protein-mediated oligomerization. Sci STKE 2003; 2003: PL1.

31. Vanden Berghe T, van Loo G, Saelens X, Van Gurp M, Brouckaert G, Kalai M et al. Differential signaling to apoptotic and necrotic cell death by Fas-associated death domain protein FADD. J Biol Chem 2004; 279: 7925–7933.

32. Dillon CP, Oberst A, Weinlich R, Janke LJ, Kang TB, Ben-Moshe T et al. Survival function of the FADD-CASPASE-8-cFLIP(L) complex. Cell Rep 2012; 1: 401–407.

33. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. Cell 2006; 126: 995–1004.

34. Newton K, Dugger DL, Wickliffe KE, Kapoor N, de Almagro MC, Vucic D et al. Activity of protein kinase RIPK3 determines whether cells die by necroptosis or apoptosis. Science 2014; 343: 1357–1360.

35. Wu X-N, Yang Z-H, Wang X-K, Zhang Y, Wan H, Song Y et al. Distinct roles of RIP1–RIP3 hetero- and RIP3–RIP3 homo-interaction in mediating necroptosis. Cell Death Differ 2014; e-pub ahead of print 6 June 2014; doi:10.1038/cdd.2014.77.

36. Feoktistova M, Geserick P, Panayotova-Dimitrova D, Leverkus M. Pick your poison: the Ripoptosome, a cell death platform regulating apoptosis and necroptosis. Cell Cycle 2012; 11: 460–467.

37. Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang JG, Alvarez-Diaz S et al. The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. Immunity 2013; 39: 443–453.

Chapter 4. RIPK3 ACTIVATION LEADS TO CONTINUED CYTOKINE SYNTHESIS AFTER LOSS OF CELL MEMBRANE INTEGRITY

This chapter is based on the following manuscript:

Susana L. Orozco, Brian P. Daniels, Nader Yatim, Michelle N. Messmer, Annelise G. Snyder, Pooja Jain, Stephen W.G. Tait, Matthew L. Albert, and Andrew Oberst. RIPK3 activation leads to continued cytokine synthesis after loss of cell membrane integrity. *Submitted manuscript.*

Necroptosis is a form of programmed cell death that is defined by activation of the kinase RIPK3, and subsequent cell membrane permeabilization by the effector MLKL. In addition to triggering cell death, RIPK3 activation can promote immune responses through the production of cytokines and chemokines, but how this function of RIPK3 is temporally coordinated with necroptotic cell death is not clear. Here, we report that cytokine production continues within necroptotic cells even after they have lost cell membrane integrity and fully committed to die. This continued production of inflammatory mediators occurs at the level of mRNA translation, and proteins produced in "dead" cells are secreted via an ER-dependent mechanism. The continued translation of cytokines by cellular corpses contributes to necroptotic cell uptake by innate immune cells, as well as priming of adaptive immune responses to antigens associated with necroptotic corpses. These findings indicate that necroptosis represents a program in which cell death and production of inflammatory mediators are coordinated to optimize the immunogenicity of necroptotic cells.

4.1 Introduction

Programmed cell death can occur via several pathways, and the way a cell dies influences the immune response it generates (*1*). In addition to apoptosis, which is generally considered immunologically silent, cells can undergo death via the lytic processes of pyroptosis or necroptosis. Pyroptosis and necroptosis can occur in response to pathogenic infection, and are associated with inflammation and adaptive immunity (*2*). It is now appreciated that these cell death programs influence the immune system through the active generation of

immunostimulatory signals during cell death. The activating cleavage of IL-1β and IL-18 by caspase-1 that accompanies pyroptosis is a well-described example of this paradigm (*3, 4*).

Necroptosis is a distinct cell death program, triggered in response to viral infection through formation of a cytosolic complex containing the kinases RIPK1 (*5, 6*) and RIPK3 (*7-9*), and subsequent phosphorylation of the membrane-disrupting pseudokinase MLKL (*10-13*). We recently demonstrated that necroptosis engages the immune system through the generation of a RIPK1 and NF-κB-dependent transcriptional response that accompanies necroptotic cell death (*14*). This transcriptional signaling leads to an increase in cross-priming of T cells responsive to antigens derived from necroptotic cells. However, this finding raised the question of how a necroptotic cell is able to actively generate immunostimulatory cytokines while committing to the terminal process of cell death.

Here, we demonstrate that cells undergoing necroptosis, which have irreversibly lost plasma membrane integrity, nonetheless maintain their ability to synthesize cytokines and chemokines. This process involves continued mRNA translation in cellular "corpses," proceeds via an endoplasmic reticulum (ER)-dependent mechanism, enhances the uptake of necroptotic cell-derived material, and contributes to the immunogenicity of necroptotic cell-derived antigens, *in vivo*.

4.2 RESULTS

RIPK3 activation leads to cytokine synthesis that continues after loss of cell membrane integrity

To study the effects of RIPK3 activation, we previously described a system by which RIPK3 can be activated directly, independent of upstream receptor signaling (*15*). Briefly, we

created a chimeric form of RIPK3, composed of murine RIPK3 fused to tandem copies of the dimerizable domain $FKBP^{F36V}$. We term the resulting chimeric, activatable RIPK3 construct "RIPK3-2xFV" (Fig. 1A). Consistent with previous reports from ourselves and others (*14, 15*), clonal populations of NIH-3T3 cells expressing RIPK3-2xFV underwent rapid and uniform necroptosis, as measured by the uptake of the cell-impermeable DNA-binding dye Sytox Green upon addition of the small-molecule dimerizer drug termed "AP1." We observed that >99% of cells were positive for Sytox Green 3 hours after addition of AP1 (Fig. 1B). The robust induction of necroptosis by this system was further confirmed by three other commonly used assays for cell death: kinetic imaging using an IncuCyte system, release of lactate dehydrogenase (LDH), and CellTiter-Glo viability assay (Fig. S1A).

Also consistent with our previous studies, we found that RIPK3 activation via this system led to the production of inflammatory cytokines accompanying necroptosis (*14*). Unexpectedly, kinetic analysis revealed that the quantities of pro-inflammatory cytokines and chemokines present in the supernatants of necroptotic cells continued to increase up to 9 hours post-RIPK3 activation, well after these cells had lost membrane integrity and were, by all traditional measures, dead (Fig. 1B-C and S1A).

These findings implied that necroptotic cells may continue to produce inflammatory cytokines even after the irreversible loss of cell membrane integrity and commitment to cell death. To test this idea, we assessed the effect of inhibiting transcription or translation, either before RIPK3 activation or after loss of cell membrane integrity. We found that addition of either actinomycin D (ActD) or cycloheximide (CHX), which inhibit transcription and translation respectively, to cells prior to RIPK3 activation abolished cytokine production during necroptosis, consistent with our previous findings that this production represents *de novo*

synthesis following activation of RIPK3 and RIPK1 (Fig. 1D) (*14*). We next activated RIPK3, then waited until the 3 hours, at which >99% of cells have lost membrane integrity (Fig. 1B). While addition of ActD to these cellular "corpses" did not alter continued accumulation of cytokines, addition of CHX abrogated further increase in cytokines levels in cellular supernatants (Fig. 1E). This finding implies that protein translation, but not transcription, continues in necroptotic "corpses" following the loss of membrane integrity, allowing for continued production and accumulation of inflammatory cytokines.

A recent study demonstrated that the ESCRT-III machinery can act downstream of MLKL to regulate necroptotic death and sustain cell survival (*16*). Given this finding, we sought to confirm that uptake of Sytox Green was an accurate marker of irreversible loss of cell membrane integrity, and that Sytox Green-positive cells could not recover and regain viability. To do this, we titrated the strength of RIPK3 activation in our RIPK3-2xFV cell line using a competitive inhibitor of the dimerization ligand (Washout Ligand) in the presence of Sytox Green, waited 24 hours, then treated cells with another cell-impermeable dye, Yoyo-3 (Fig. S1B). We hypothesized that if cells transiently permeabilized their membranes and then recovered, we would detect a population of cells that became marked with Sytox Green, then recovered membrane integrity sufficiently to exclude Yoyo-3. However, we did not observe this; all cells that lost membrane integrity upon RIPK3 activation (Sytox Green-positive) remained permeable 24 hours later (Yoyo-3-positive) (Fig. S1C). Consistently, we found that after 3 hours of RIPK3 activity, the potential for clonogenic outgrowth was irrecoverably lost (Fig. S1D). These data indicate that loss of cell membrane integrity, as indicated by uptake of Sytox Green, is an irreversible event from which cells cannot recover.

Together, these findings support the hypothesis that the synthesis of inflammatory cytokines and chemokines in necroptotic cells continues even after cell membrane integrity is irreversibly lost and cells appear “dead” by several commonly accepted assays. This continued cytokine synthesis occurs via ongoing translation of existing mRNA transcripts. We term this phenomenon “zombie translation.”

Figure 1

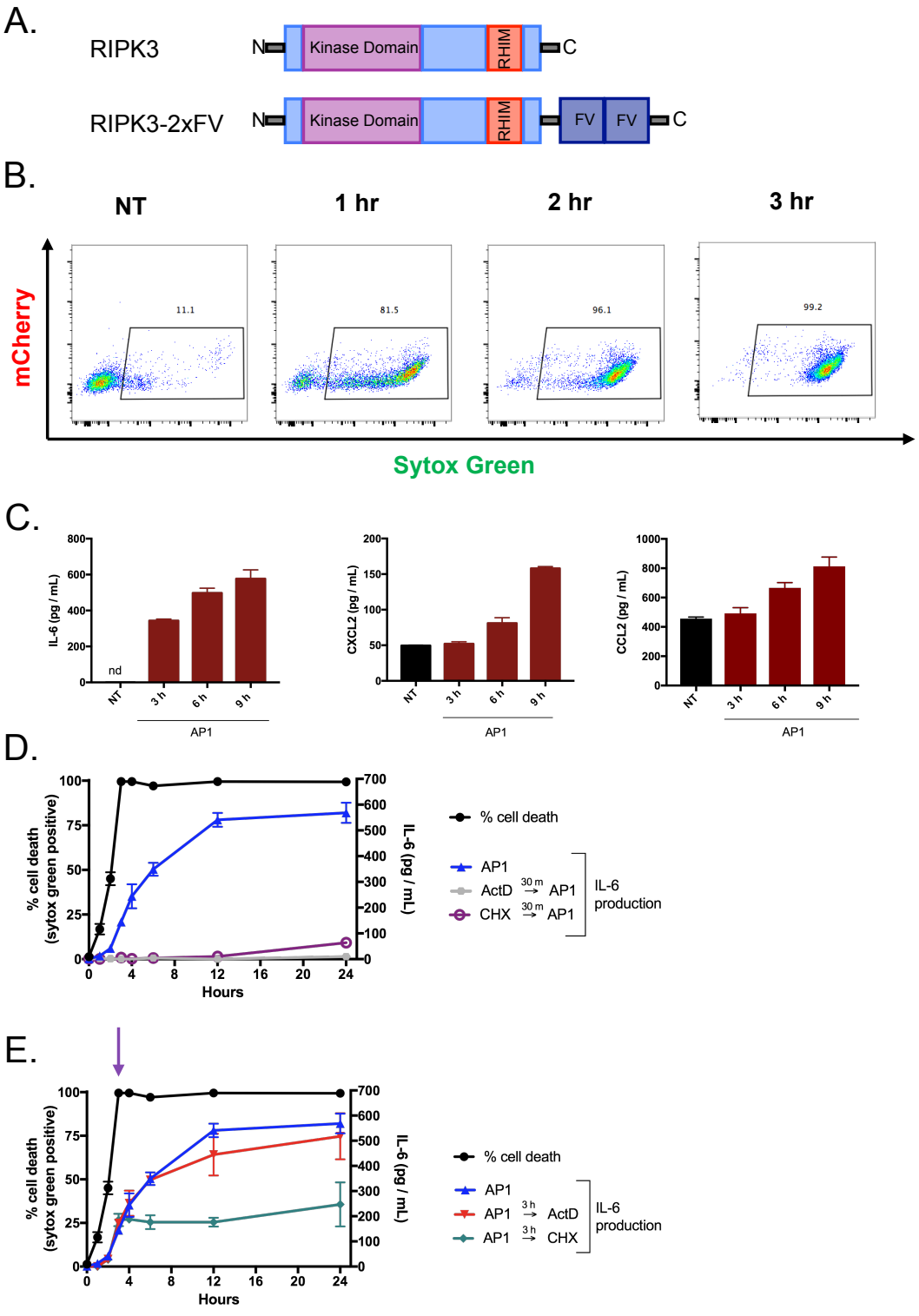

A.
RIPK3
N
Kinase Domain
RHIM
C
RIPK3-2xFV
FV
FV
B.
NT
1 hr
2 hr
3 hr
mCherry
11.1
81.5
96.1
99.2
Sytox Green
C.
IL-6 (pg / mL)
nd
CXCL2 (pg / mL)
CCL2 (pg / mL)
NT
3 h
6 h
9 h
AP1
D.
% cell death (sytox green positive)
IL-6 (pg / mL)
Hours
% cell death
AP1
ActD 30 m → AP1
CHX 30 m → AP1
IL-6 production
E.
% cell death (sytox green positive)
IL-6 (pg / mL)
Hours
% cell death
AP1
AP1 3 h → ActD
AP1 3 h → CHX
IL-6 production

Figure 4.1. RIPK3 activation leads to ongoing translation even after cell membrane permeabilization.
(A) Schematic representation of RIPK3 and the chimeric RIPK3 construct used in this study. (B) NIH-3T3 fibroblasts expressing RIPK3-2xFV were treated with dimerizer drug (AP1) for the indicated times and analyzed for the uptake of the cell impermeable dye (Sytox Green) by flow cytometry. (C) ELISA analysis of various cytokines in the supernatant of NIH-3T3 cells induced to undergo necroptosis. (D) Kinetic comparison of Sytox Green uptake (black line) versus *de novo* IL-6 production. Cells were pre-treated with either actinomycin D (ActD) or cycloheximide (CHX) 30 mins before AP1 treatment. (E) NIH-3T3 cells were induced to undergo necroptosis (AP1 treatment), and were then treated with ActD or CHX at a time where all cells had lost membrane integrity (purple arrow, 3 hrs). (D and E) IL-6 in the supernatant was quantified by ELISA. Data are representative of at least three independent experiments.

Zombie translation occurs in necroptotic cell "corpses"

We next sought to understand whether zombie translation occurred within necroptotic corpses, or in an extracellular compartment such as an exosome or the cell culture supernatant itself. To address this question, we induced necroptosis in NIH-3T3 cells, and once cells had lost membrane integrity, we separated supernatants from corpses by centrifugation and analyzed further cytokine synthesis from each component (Fig. 2A). We observed that isolated corpses, but not their supernatants, had the same increase in IL-6 levels as unseparated necroptotic cell cultures. Addition of CHX to cell corpses following isolation from supernatants inhibited further cytokine synthesis (Fig. 2B). These data suggest that zombie translation occurs within the necroptotic corpse.

To better understand the source of zombie translation, we added the membrane-impermeable translation inhibitor gelonin to necroptotic corpses, then measured cytokine levels. We hypothesized that upon loss of cell membrane integrity, gelonin would gain access to the cytosol and block translation. Surprisingly, we found that while gelonin efficiently inhibited translation in an *in vitro* translation assay, addition of gelonin to necroptotic corpses did not abolish their ongoing translation, despite their fully permeabilized plasma membranes (Fig. 1C).

We hypothesized that perhaps some membrane-bound organelle may protect the translation machinery, thereby denying access to gelonin. To test this, we treated cells with the mild detergent digitonin, at a concentration that triggers permeabilization of all cellular membranes. While digitonin did not directly affect translation in an *in vitro* assay (Fig. S2A), adding digitonin to necroptotic corpses abolished zombie translation (Fig. 2D). This led us to speculate that aspects of the ER-Golgi transport network might remain intact in necroptotic corpses. To test this, we treated corpses with brefeldin A (BfA), an inhibitor of protein transport from the ER to the Golgi apparatus. BfA treatment lead to a reduction in zombie cytokines in the supernatant (Fig. 2E). Together, the data suggest that zombie cytokine translation occurs within necroptotic corpses, requires intact membrane compartments within those corpses, and relies on the ER-to-Golgi transition for secretion.

The observation of continued translation from necroptotic corpses could be explained by the presence of an undetected population of viable cells. Given our findings indicating that translation occurs within cellular corpses, we devised an assay allowing us to quantify zombie translation within individual necroptotic cells. To do this, we induced necroptosis in NIH-3T3 cells, then briefly pulsed them with a cell-impermeable dye to specifically mark only cells that had lost membrane integrity at this time point. These cellular corpses were then incubated with BfA to allow the accumulation of cytokines within the ER; these were then quantified using standard intracellular cytokine staining and flow cytometry (Fig. 2F). By analyzing only cells marked by the pulse of cell-impermeable dye, we were thereby able to quantify cytokine accumulation in individual cells after the confirmed loss of membrane integrity. We found that these cells showed a significant increase in accumulation of intracellular CCL2 after loss of membrane integrity, and that this effect could be inhibited by blocking translation (Fig. 2G).

Figure 2

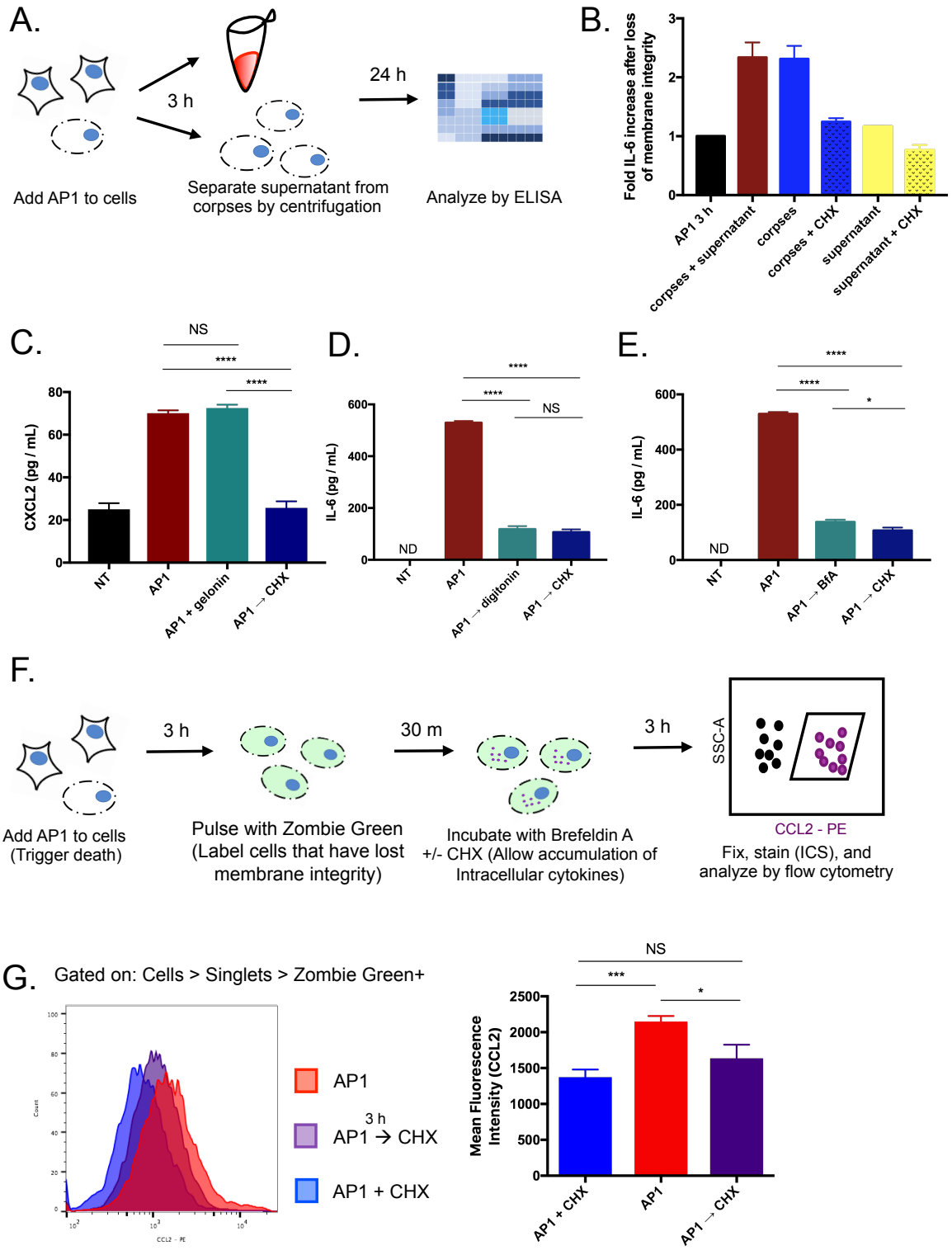


Figure 4.2. Zombie cytokine translation occurs within corpses.

(A) Experimental schematic for (B). (B) NIH-3T3 cells were treated as in (A), and supernatants analyzed by ELISA. Values are plotted as fold-increase in IL-6 after Sytox Green uptake (3 hrs). (C, D, and E) NIH-3T3 cells were induced to undergo necroptosis (AP1 treatment), or were treated with AP1 +/- gelonin (C), and then treated with either digitonin (D) or brefeldin A (E) after 3 hours (→). The concentration of cytokine in the supernatant was quantified at 9 hrs post AP1-addition. (F) Experimental schematic for (G). (G) Cells were treated as in (F), and intracellular CCL2 concentration was quantified by flow cytometry. The gating for both histogram (left panel) and MFI (right panel) is: all cells > singlets > Zombie Green^{+}. All data are representative of at least three independent experiments. ****$p < 0.0001$, ***$p < 0.001$, *$p < 0.05$. NS, not significant. N.D., not detected.

Zombie translation of cytokines is associated with the ER in necroptotic corpses

We next sought to directly visualize protein translation in necroptotic cell corpses. To do this, we made use of puromycin, which is covalently incorporated into growing polypeptide chains by translating ribosomes (*17*). These puromycilation events can be detected and visualized using a monoclonal antibody to puromycin (Fig. 3A). We first pulsed corpses with a cell-impermeable dye to confirm loss of membrane integrity, then pulsed with puromycin, which would be incorporated only if translation was ongoing. We found that cells in which membrane integrity was disrupted via necroptosis nonetheless incorporated puromycin into newly synthesized polypeptides, consistent with zombie translation in "dead" cells. Notably, puromycilated polypeptides co-localized with the ER marker ERp72 (Fig. 3B), consistent with the idea that the ER is the site of ongoing translation in necroptotic cells. Additionally, we did not observe puromycin incorporation (and thus, ongoing translation) in cells in which intracellular membranes were disrupted by digitonin, or in which translation was blocked by CHX (Fig. S3A). Also consistent with a role for the ER in zombie translation, we found that treatment of necroptotic corpses with the ER disruptor thapsigargin abrogated continuing cytokine production (Fig. 3C).

Activated MLKL, the executioner molecule of necroptosis, has been shown to translocate to the plasma membrane and disrupt membrane integrity, though imaging studies have indicated that most activated MLKL localizes to the plasma membrane (*16, 18-20*). Given our finding of ongoing translation following MLKL-mediated plasma membrane disruption, and our implication of the ER in this process, we hypothesized that activated MLKL does not disrupt this organelle, and that this property allows for zombie translation.

We sought to directly test the idea that selective permeabilization of the plasma membrane, but not the ER, by activated MLKL is required for zombie translation. To do this, we engineered an MLKL construct targeted to the cytoplasmic surface of the ER, though addition of a sequence derived from the C-terminus of cytochrome b5 (*21*); we reasoned that forced ER disruption by this MLKL construct (MLKL-ER_{cb5}) would abrogate zombie translation. As expected, expression of this ER-restricted MLKL construct in cells expressing endogenous MLKL did not affect the kinetics of plasma membrane permeabilization upon RIPK3 activation (Fig. 3D). However, production of both IL-6 and CXCL2 after loss of plasma membrane integrity was curtailed in cells expressing MLKL-ER_{cb5} (Fig. 3E). These data suggest that upon RIPK3 activation, MLKL may leave the ER intact, allowing for continued translation of cytokine mRNA; upon enforced targeting of MLKL to the ER, zombie cytokine production is disrupted.

Figure 3

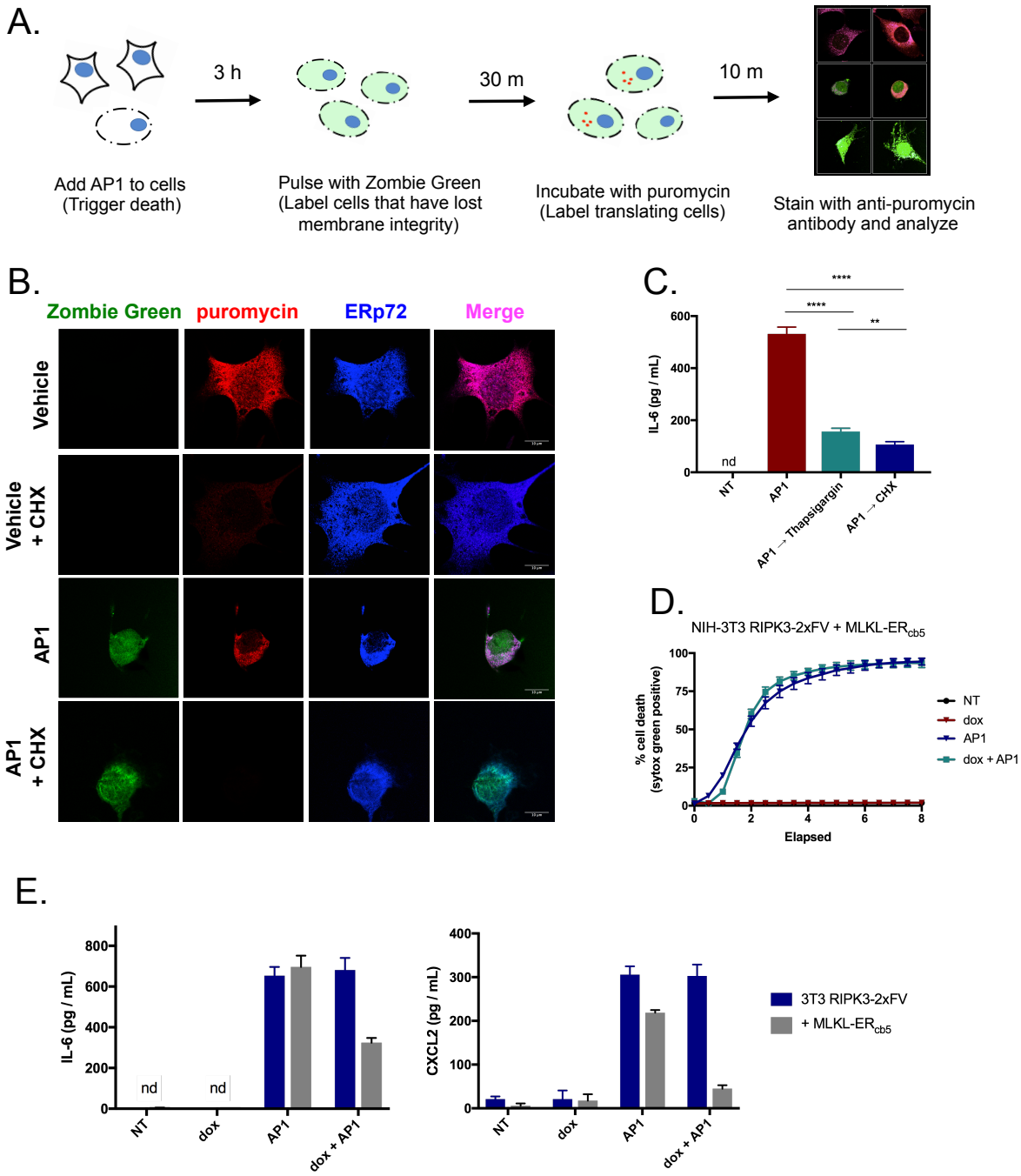


Figure 4.3. Zombie translation is associated with the ER in necroptotic corpses. (A) Experimental schematic for (B). (B) NIH-3T3 cells were treated as in (A) and analyzed by microscopy. Representative images for each condition are shown. Scale bar = 10 μm. (C) Cells were induced to undergo necroptosis (AP1 treatment), and then treated with thapsigargin after 3 hours (→). IL-6 in the supernatant was quantified at 9 hrs post-AP1 treatment. (D) Death kinetics

of NIH-3T3 cells expressing an inducible ER-targeted version of MLKL (MLKL-ER_{cb5}) were analyzed using IncuCyte imaging. (E) NIH-3T3 cells expressing MLKL-ER_{cb5} or the parental line were pre-treated with doxycycline for 4 hours, then treated with AP1 to induce necroptosis. IL-6 and CXCL2 in the supernatant were quantified by ELISA at 18 hrs post-AP1 addition. Data in (B), (D), and (E) are representative of two independent experiments; data in (C) are representative at least three independent experiments.

Viral infection leads to necroptosis and zombie translation

Several viruses have been shown to induce RIPK3-dependent death through activation of the nucleotide sensor DAI/Zbp1 (*22-25*), so we sought to determine whether zombie translation occurs following necroptosis triggered via this pathway. In order to test this, we used a mutant strain of murine cytomegalovirus (MCMV M45ΔRHIM) that triggers robust necroptosis (*22*). As expected, SVEC4-10 endothelial cells underwent RIPK3-dependent cell death upon infection with this virus (Fig. 4A and fig. S4A). To measure the induction of zombie translation in these cells, we pulsed SVEC 4-10 cells with a membrane-impermeable dye 6 hours after infection, then measured subsequent accumulation of cytokines in cells that had lost membrane integrity using intracellular cytokine staining, analogous to the experiment shown in Fig. 2F-G. Flow cytometric analysis revealed that cytokines continued to accumulate in necroptotic corpses after virus-induced necroptosis, and that this effect was abrogated by blocking translation after loss of membrane integrity (Fig. 4B and fig. S4B).

To further characterize the induction of virus-induced zombie translation, we used puromycin incorporation (analogous to Fig. 3B) to label translating ribosomes in SVEC 4-10 cells killed by MCMV-induced necroptosis. We observed that puromycin incorporation co-localized with ERp72 in cells that had lost plasma membrane integrity, and that this puromycin incorporation could be blocked by inhibiting translation, or by disrupting intracellular

membranes using digitonin (Fig. 4C). Together, these data indicate that zombie translation occurs following necroptosis induced by viral infection.

Figure 4

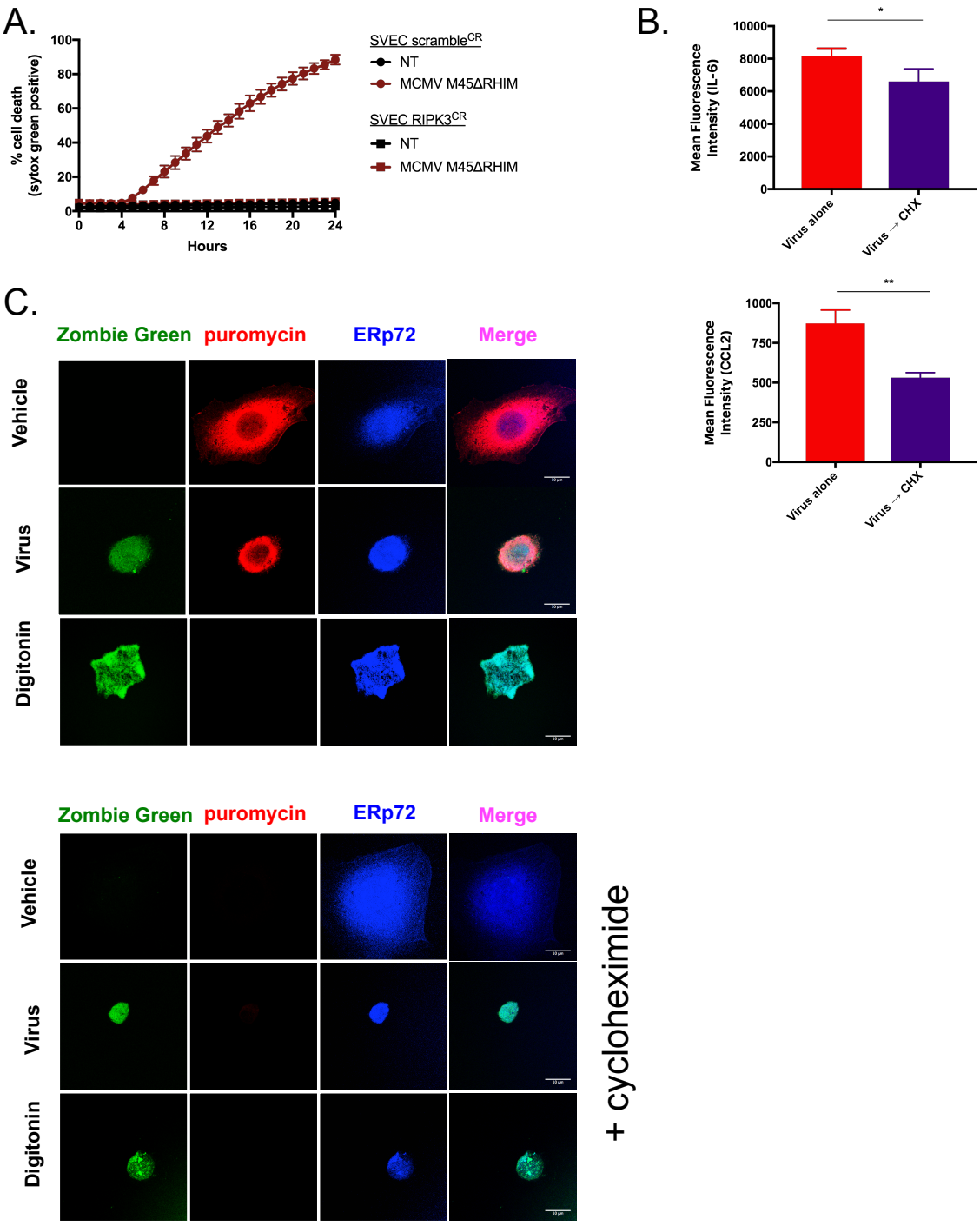


Figure 4.4. Infection with MCMV M45ΔRHIM leads to necroptosis and zombie translation. (A) SVEC4-10 cells with or without RIPK3 (scramble^{CR} vs. RIPK3^{CR}, using the CRISPR/Cas9 system) were infected with MCMV M45ΔRHIM at an MOI of 1, and death kinetics were analyzed via IncuCyte bioimaging. (B) SVEC4-10 cells were infected with MCMV M45ΔRHIM at an MOI of 1 for 6 hours, then treated as in Fig. 2F. Intracellular cytokines were quantified by flow cytometry. Gating: cells > singlets > Zombie Green^{+}. (C) SVEC4-10 cells were infected at an MOI of 1 for 6 hours, then treated as in Fig. 3A, with or without cycloheximide. Cells were imaged by microscopy, and representative images for each condition are shown. Scale bar = 10 μm. Data in (A) and (B) are representative of at least three independent experiments; data in (C) are representative of one independent experiment.

Cytokine and chemokine mRNAs are associated with translating polyribosomes in necroptotic corpses

Our previous work has shown that upon RIPK3 activation, cells upregulate a number of pro-inflammatory mRNAs, and this burst of transcription accompanies necroptotic cell death (*14*). Given our observation that translation occurs in necroptotic corpses that have lost cell membrane integrity, and we sought to characterize the targets of this ongoing translation. To do this, we subjected live cells and necroptotic corpses to polyribosome (polysome) analysis (*26*). Briefly, NIH-3T3 cells were induced to undergo necroptosis for 4 hours, and corpse lysates were layered over a sucrose gradient and ultracentrifuged, causing mRNAs associated with multiple ribosomes (translating fraction) to separate from free mRNAs or those associated with monosomes (non-translating fractions). Gradients were then fractionated while continuously monitoring absorbance at 254 nm, giving rise to polysome profiles (Fig. 5A). In these experiments, CHX is added to freeze ribosomes to mRNA, while EDTA, which dissociates ribosomes from mRNA, is used as a negative control. Using this approach, untreated cells or cells treated with TNFα displayed the expected profile of monosomes and polysomes (Fig. 5B). While necroptotic corpses did not have a clear polysome peak, we were able to detect abundant cytokine and chemokine transcripts by qPCR in the corresponding fractions (Fig. 5C). Interestingly, cells that had lost membrane integrity were more likely to translate cytokine or

chemokine transcripts (relative to the housekeeping gene GAPDH), as compared to non-treated or TNFα-treated cells (Fig. 5B). For comparison, we also quantified the translation of CCL5, a gene we have seen induced by TNFα treatment but not RIPK3 activation (Fig. 5D), and found that it was not associated with ribosomes in necroptotic corpses. Notably, input lysates (taken prior to fractionation) revealed similar and expected total transcript levels between samples that were treated with either CHX or EDTA (Fig. S5A); however, EDTA treatment abolished the association of mRNAs with polysome fractions, as expected (Fig. S5B). These data indicate that upon RIPK3 activation, necroptotic cells upregulate cytokine and chemokine transcripts, and that their translation occurs even after loss of cell membrane integrity.

Figure 5

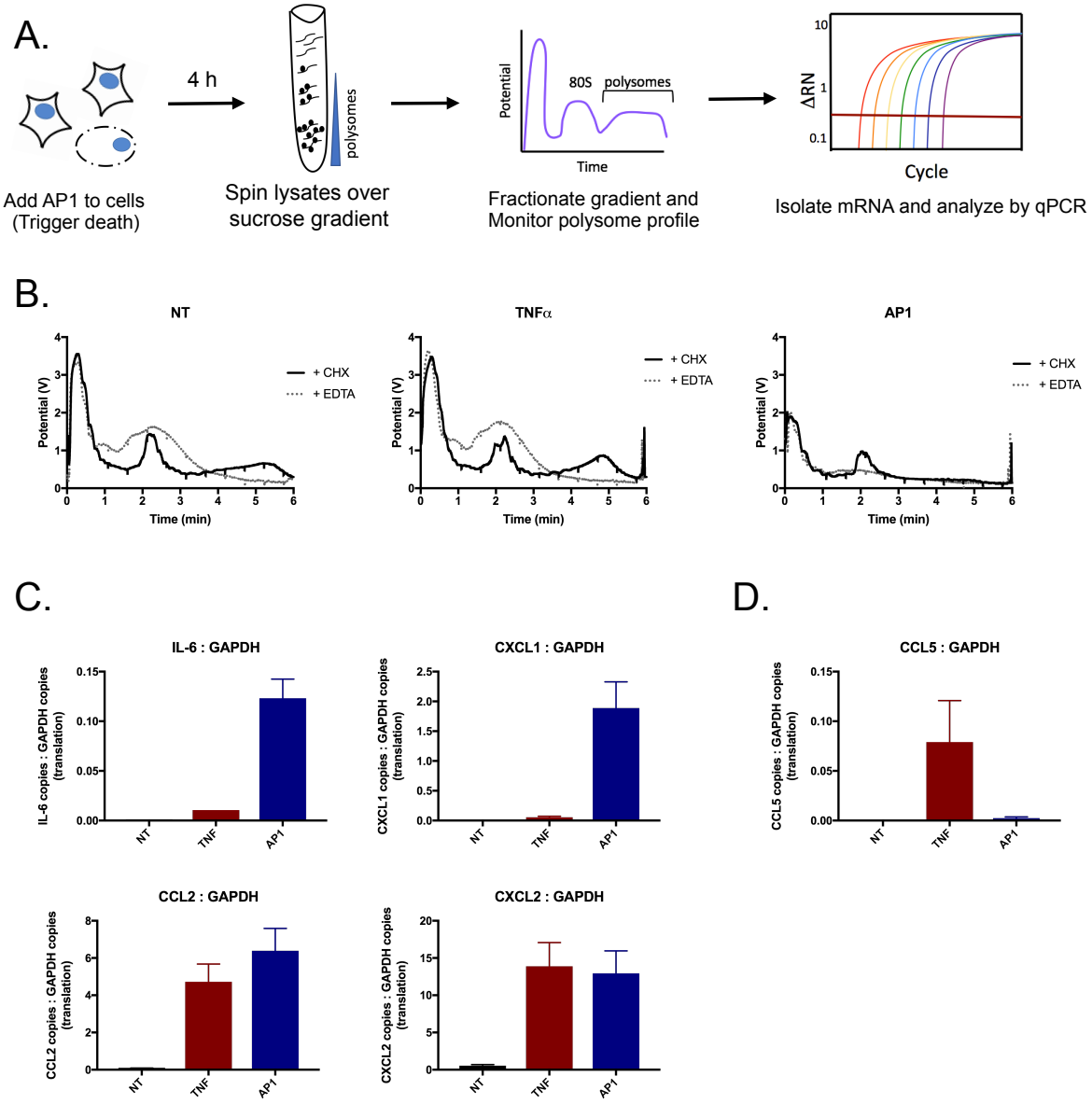


Figure 4.5. Necroptotic cells that have lost membrane integrity preferentially translate cytokine and chemokine mRNA.

(A) Experimental schematic for (B) and (C). NIH-3T3 cells were treated with either vehicle, TNFα, or AP1 for 4 hours, then treated with CHX (to freeze ribosomes to mRNA). Cell lysates were then spun over a sucrose gradient to separate free ribosomes from translating polyribosomes. (B) Gradients were fractionated while continuously monitoring absorbance at 254 nm, giving rise to polysome profiles. (C and D) RNA was extracted from the fractions corresponding to polysome peaks from (B), and mRNA transcripts were analyzed by qPCR. Graphs represent the ratio of total cytokine transcripts to a housekeeping gene (GAPDH) being

actively translated in each condition. All data are representative of three independent experiments.

Zombie translation influences myeloid cell migration and promotes CD8$^+$ T cell responses to necroptotic cell-derived antigen

We have previously shown that cytokines and chemokines produced upon activation of RIPK3 can promote both inflammatory and adaptive immune responses *in vivo (14)*. Given this, we sought to test whether continued cytokine and chemokine production could promote immune responses to necroptotic cell corpses. We first tested the effect of zombie translation on the uptake and trafficking of necroptotic cell material from a peripheral tissue to the draining lymph node. To do this, we loaded NIH-3T3 cells with green-fluorescent nanoparticles (FluoSpheres), which remain associated with necroptotic corpses after loss of membrane integrity (Fig. S6A) and are not degraded upon phagocytosis of dead cells. We then injected corpses (verified >95% permeabilized by flow at time of injection, *data not shown*) into the footpads of mice, then analyzed FluoSphere-positive phagocytes in the draining lymph node (dLN, popliteal) 24 hours later, as a measure of the uptake and trafficking of necroptotic cell-derived material (Fig. 6A). To assess the effect of zombie translation on this process, we also tested the effect of transiently pulsing necroptotic corpses with emetine, an irreversible translation inhibitor (*27*) (Fig. S6B). We observed a similar number of FluoSphere-positive myeloid cells in the dLNs of mice that received either actively dying cells (cells induced to undergo necroptosis *in vivo*) or corpses; however, there were significantly fewer FluoSphere-positive cells in the dLNs of those animals that received emetine-pulsed corpses (Fig. 6B). We also saw a decreased number of FluoSphere-positive migratory DCs (as defined by CD11c$^+$ MHCII^{hi} myeloid cells) in the dLNs of animals

that received emetine-treated corpses relative to animals that received either corpses in which zombie translation was occurring, or when cells were killed *in vivo* (Fig. 6C). However, we did not see a difference in the number of total myeloid cells present in the dLNs across treatment groups (Fig. S6C). These data suggest that zombie translation is necessary for myeloid cells to efficiently take up necroptotic cells and traffic necroptotic cell-derived material to the dLN.

Previously, we demonstrated that NF-κB activity downstream of RIPK3 activation is required for the cross-priming of $CD8^+$ T cell responses against antigen derived from necroptotic cells (*14*). Given our observation that necroptotic corpses continue translating pro-inflammatory mediators even after loss of cell membrane integrity, we hypothesized that zombie translation contributes to the cross-priming potential of these corpses *in vivo*. To address this, we immunized C57BL/6 mice with cells containing membrane-bound ovalbumin (mOVA) that underwent necroptosis *in vivo*, with corpses undergoing zombie translation, or with emetine-pulsed corpses in which ongoing translation was abrogated. As all these cells expressed mOVA, we could track OVA-specific $CD8^+$ T cell responses nine days later as a measure of the cross-priming response elicited by each dying cell vaccination treatment. Mice that received either actively dying necroptotic cells or corpses showed efficient cross-priming, and had no significant difference in the number of OVA-specific $CD8^+$ T cells (as measured by SIINFEKL-H2Kb tetramer) or activated ($CD44^+CD62L^-$) $CD8^+$ T cells; however, mice that received emetine-treated corpses had significantly fewer antigen-specific cells and activated $CD8^+$ T cells (Fig. 6D). This finding suggests that continued zombie translation is necessary to elicit a robust cross-priming response *in vivo.* Together, these data indicate that the continued production of inflammatory cytokines and chemokines by necroptotic cell corpses promotes the trafficking of

necroptotic cell-derived antigens to draining lymph nodes and the initiation of T cell responses to dying cell-derived antigen.

Figure 6

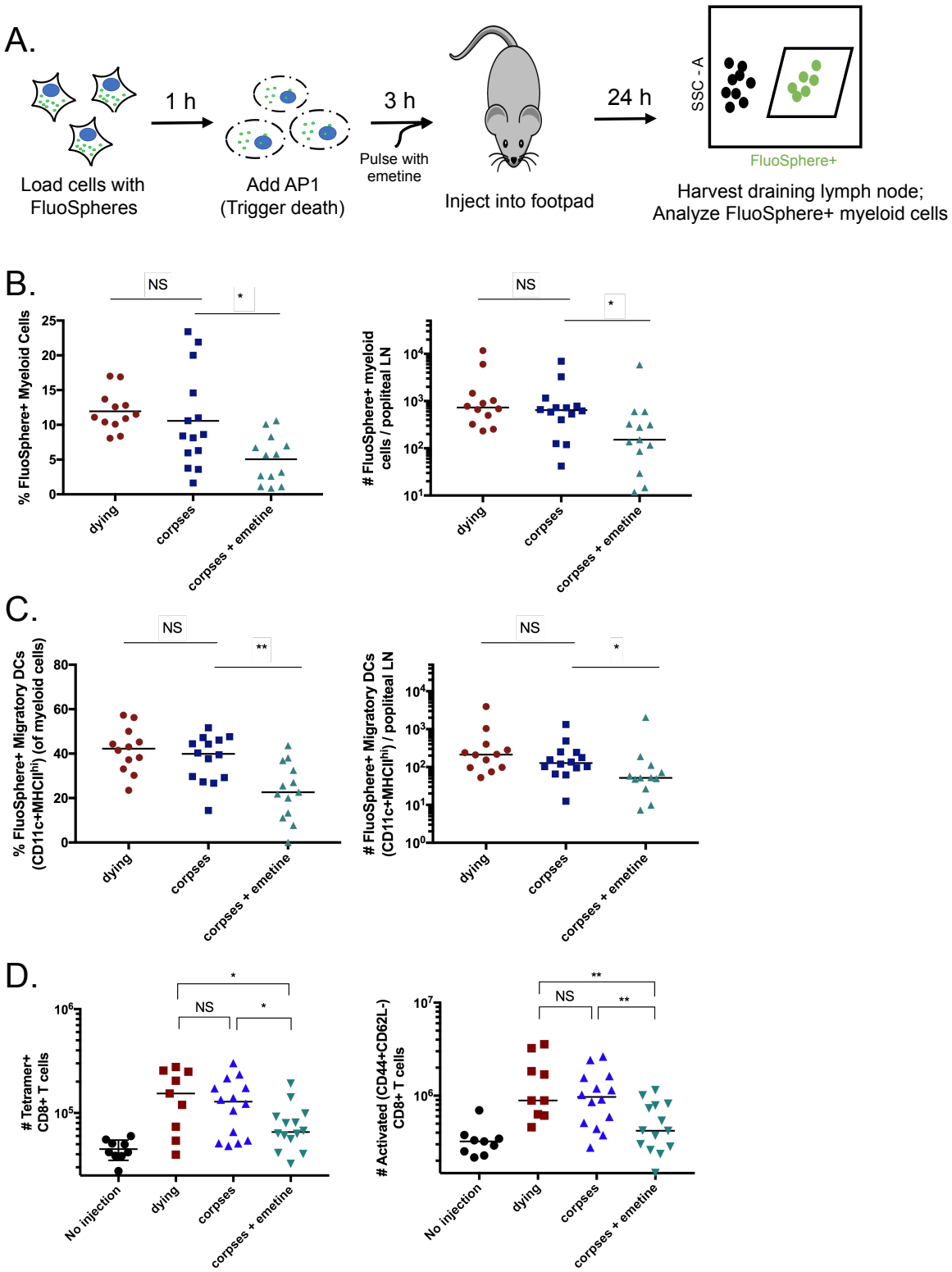

A.
1 h
3 h
Pulse with emetine
24 h
SSC - A
FluoSphere+
Load cells with FluoSpheres
Add AP1 (Trigger death)
Inject into footpad
Harvest draining lymph node; Analyze FluoSphere+ myeloid cells
B.
% FluoSphere+ Myeloid Cells
# FluoSphere+ myeloid cells / popliteal LN
C.
% FluoSphere+ Migratory DCs (CD11c+MHCII^hi) (of myeloid cells)
# FluoSphere+ Migratory DCs (CD11c+MHCII^hi) / popliteal LN
D.
Tetramer+ CD8+ T cells
Activated (CD44+CD62L-) CD8+ T cells
NS
dying
corpses
corpses + emetine
No injection

Figure 4.6. Zombie translation influences myeloid cell trafficking and cross-priming potential necroptotic cell-derived antigen.
(A) Experimental schematic for (B) and (C). (B and C) Percentage and total number of FluoSphere-positive myeloid cells ($CD3^-B220^-CD11b^+$)(B) or migratory dendritic cells ($CD11c^+MHCII^{hi}$) (C) in the draining lymph node (popliteal) of mice, 24 hours after injection in the footpad. (D) NIH-3T3 cells expressing membrane-bound OVA (either actively dying *in vivo*, corpses, or corpses pulse-treated with emetine) were injected into C57Bl/6 mice. 9 days later, OVA-specific and activated ($CD44^+CD62L^-$) $CD8^+$ T cell responses were measured by flow cytometry. Data in (B), (C), and (D) each represent two independent experiments combined. *$p < 0.05$, **$p < 0.01$, NS, not significant.

4.3 DISCUSSION

The data presented here indicate that RIPK3 activation leads not only to necroptotic cell death, but also the production of inflammatory cytokines and chemokines which continues after loss of plasma membrane integrity. We observed this phenomenon, which we termed zombie translation, downstream of either direct RIPK3 activation or necroptosis in response to viral infection. This phenomenon contrasts with apoptotic cell death, during which protein synthesis is actively antagonized through caspase-mediated destruction of the translation machinery (*28*). Indeed, a previous report indicated that disruption of the protein synthesis machinery is a feature of apoptosis that does not occur during necrosis (*29*). Our data imply that continued protein synthesis by necroptotic "corpses" may be an evolved response that allows coordination of cellular destruction with initiation of an inflammatory response.

There have been contradictory reports regarding the mechanism and location of MLKL's membrane disrupting activity. While some studies have suggested that MLKL targets and disrupts the integrity of both intracellular and plasma membranes (*30*), others, including recent high-content imaging approaches, have indicated that active MLKL is restricted only to the plasma membrane (*16, 18-20*). Our work supports the idea that MLKL leaves the ER network intact during necroptosis, and this allows for zombie translation; in fact, we demonstrate here

that forced targeting of MLKL to the ER disrupts zombie translation without affecting the execution of MLKL-dependent cell death.

Previous work has suggested that necroptosis can actually dampen inflammatory responses by eliminating cells that would otherwise continue cytokine synthesis in response to TNFα or LPS (*31*). Our data do not contradict this idea, as the total magnitude of inflammatory mediators produced by a live cell in response to these stimuli would outstrip the "zombie" production we observe. Nonetheless, our data suggest that zombie cytokine production is an important contributor to the inflammatory and immune response to necroptotic cells, and that this mechanism renders necroptotic cells more immunostimulatory than cells dying by apoptosis or unprogrammed necrosis.

We speculate that, during the early stages of a viral infection, the necroptotic program evolved to eliminate the virus's replicative niche by destroying the plasma membrane, while continuing cytokine and chemokine production to alert the immune system and recruit relevant immune cells to the site of infection. A further, non-exclusive possibility is that viral proteins can also be translated in necroptotic corpses, thereby loading the necroptotic corpse with viral antigen. As we have previously shown that antigens derived from necroptotic cells more readily cross-prime $CD8^+$ T cells (*14*), this mechanism could contribute to the induction of antiviral immune responses.

Together, our findings indicate that the necroptotic program involves the coordination of cell death with continued cytokine synthesis, and that this property contributes to the inflammatory and immunogenic nature of necroptosis.

4.4 MATERIALS AND METHODS

Mice

Wild-type C57BL/6J mice were obtained commercially (Jackson Laboratories) and were housed under specific-pathogen-free conditions at the University of Washington. All experiments were performed in female 6-8 week-old mice, in accordance with protocols approved by the University of Washington Animal Care and Use Committee (IACUC).

Constructs and cell lines

The RIPK3-2xFV chimeric protein was constructed as previously described (*14, 15*). Briefly, full-length murine RIPK3 was cloned upstream of 2 copies of FKBP carrying the F36V mutation, herein called 'FV' domains. When two FV domains were used, the first copy contained silent mutations to prevent DNA recombination. These RIPK3-FV fusion proteins were cloned into pBabe-Puromycin retroviral vectors. This construct was transduced into NIH-3T3 cells (ATCC, CRL-1658) using standard protocols for helper-dependent retroviral transduction. Transduced cells were selected for 5 days in 1 μg/ml puromycin, then single-cell cloned to obtain a homogenous population.

MLKL-ER_{cb5} was cloned into the previously described doxycycline-inducible pSLIK-Hygromycin backbone (*14*). This construct was transduced into NIH-3T3 cells expressing RIPK3-2xFV. Transduced cells were selected for 7 days in 200 μg/ml hygromycin B (Fisher, ICN19417083).

For CRISPR/Cas9-genome editing of SVEC4-10 cells (ATCC, CRL-2181), the following guide RNA (gRNA) sequences were cloned into pRRL-Cas9-T2A-puromycin (*32*): murine non-targeting gRNA: 5'- GCGAGGTATTCGGCTCCGCG -3' (*33*); *Ripk3* gRNA: 5'-GAGGGTTCGGAGTCGTGTTC -3'. These constructs were transduced into SVEC4-10 cells using standard lentiviral transduction protocols. Transduced cells were selected for 5 days in 1 μg/ml puromycin, and validated via Western blot analysis.

All cell lines were maintained in D-MEM (Fisher, SH30022FS) supplemented with 10% FBS (Gemini Bio-Products, 100-106), 29.2 g/l glutamine (Fisher, SH3003402), 10 000 U/ml penicillin and 10 000 mg/ml streptomycin (Fisher, SV30010) and grown at 37°C in 5% CO_2.

Chemicals and compounds

Reagents used were as follows: recombinant murine TNF ✔ (Peprotech, 315-01A), AP1 dimerizer (B/B homodimerizer: Clontech, 635069), actinomycin D (Sigma-Aldrich, A1410), cycloheximide (Sigma-Aldrich, C7698), gelonin (Enzo Life Sciences, ALX-350-150-M001), digitonin (Calbiochem, 300410), brefeldin A (Fisher, 420601), puromycin dihydrochloride (Thermo Fisher, A1113803), paraformaldehyde (Fisher, 50980494), emetine dihydrochloride (EMD Millipore, 324693250MG), yellow-green FluoSpheres (Thermo Fisher, F8795), B/B Washout Ligand (Clontech, 635088).

Cell death assays

Cell death was quantified by flow cytometry on an LSRII Flow Cytometer (BD) or using a 2-color IncuCyte Zoom in-incubator imaging system (Essen Biosciences) as previously described

(*15*). Dead cells were detected by uptake of the cell-impermeable dye Sytox Green (Thermo Fisher, S7020). Cell death was also quantified by either CellTiter-Glo Luminescent Cell Viability Assay (Promega, G7570) or LDH Cytotoxicity Assay (Pierce, 88954), both according to manufacturer's instructions.

Polysome Analysis and qRT-PCR

Polysome analysis was performed as previously described (*26*). Briefly, cells were treated for 4 hours with either 20 ng/mL TNF ✔ or 500 nM AP1, and then treated with 100 μg/mL of cycloheximide or 25 mM EDTA for 5 minutes at 37°C. Cells were then pelleted and lysed in hypotonic lysis buffer (5 mM Tris-HCl pH 7.5, 2.5 mM $MgCl_2$, 1.5 mM KCl, and 1X protease inhibitor cocktail) followed by addition of 100 μg/mL cycloheximide, 2 mM DTT, 200 U/mL RNasin, 0.5% Triton-X, and 0.5% sodium deoxycholate. Lysates were clarified by spinning at max speed in a tabletop centrifuge for 5 minutes, and supernatants were loaded onto sucrose gradients (10%-50%). Samples were then ultracentrifuged using an SW40 Ti Rotor (Beckman Coulter) for 2 hours at 4°C. Gradients were fractionated using an Auto-Densi Flow Pump (Labconco) and a Foxy R2 Fraction Collector (Teledyne Isco), while continuously monitoring absorbance at 254 nm using UA-6 UV detector and LoggerPro Software (Vernier).

RNA was extracted from the fractions corresponding to polysomes using RNA Stat-60 (Amsbio, CS-110); RNA fractions from each sample were then pooled, and cDNA was made using SuperScript III Reverse Transcriptase (Thermo Fisher, 18080044). Quantitative real time PCR (qRT-PCR) analysis was performed using SYBR Green (Thermo Fisher, 11744500) and ViiA7

Real-Time PCR System (Applied Biosystems). Cytokine and *Gapdh* transcripts were quantified against a standard curve of known amounts.

Immunocytochemistry

Fluorescent immunocytochemistry was performed as previously described (*34*). Cells were fixed for 15 minutes with 4% paraformaldehyde, permeabilized with 0.1% Triton-X, and blocked with 10% goat serum. Cells were stained using primary anti-puromycin (Clone 12D10, EMD Millipore, MABE343) or anti-ERp72 (Cell Signaling Technologies, 5033S) for 1 hour at room temperature, followed by secondary goat anti-mouse AlexaFluor594 (Abcam, ab150116) or goat anti-rabbit AlexaFluor405 (Abcam, ab175654). Dead cells were visualized via staining with the fixable viability dye Zombie Green (Biolegend, 423111).

Cytokine protein quantification

Cytokine concentrations in cell culture supernatants were measured using IL-6 (Thermo Fisher, 88-7064-76), CXCL2 (Peprotech, 900-K152), or CCL2 (Thermo Fisher, 88-7391-76) ELISAs.

Western Blot

Cells were lysed in NP-40 lysis buffer (150 mM NaCl, 20 mM tris-Cl, 1 mM EDTA, 1% NP-40 at pH 7.5) with 1X Halt Protease Inhibitor Cocktail (Thermo Fisher, PI78429). 30 ¥ g of protein were run on a 4%-20% Novex Tris-Glycine mini gel (Fisher, XP04202BOX) at 125 V for 2 hours in WB running buffer (24 mM tris, 32 mM glycine, 3.5 mM SDS) and transferred onto PVDF membrane (Thermo Fisher, 88520) at 300 mAmps for 1 hour in transfer buffer (6 mM tris, 8 mM glycine, 15% methanol). Membranes were blocked in 5% dry milk in TBS-Tween20

(1%) for 30 minutes at room temperature. Membranes were incubated overnight at 4°C with primary anti-RIPK3 (Novus Biologicals, NBP1-77299), anti-V5 (Thermo Fisher, R960-25) or anti-actin (Millipore, MAB1501), followed by staining with HRP-conjugated secondary antibodies (Santa Cruz, sc-2313 and sc-2005). Membranes were developed using ECL Western Blotting Substrate (Pierce, 32209) and film.

In vitro *transcription/translation*

TNT® Quick Coupled Transcription/Translation System (Promega, L1171) was used to assess the effects of compounds on translation in a cell-free system. A construct coding for V5-tagged TEV was used with the kit, according to manufacturer's instructions. Lysates were analyzed via Western blot.

In vivo *experiments*

For migration assays: NIH-3T3 cells were loaded with 10^6 particles/cell of FluoSpheres for one hour at 37°C. Excess FluoSpheres were aspirated and cells were washed once with 1X PBS, then treated with 500 nM AP1 for 3 hours or pulsed for 15 minutes at 37°C as previously described (*14*). Corpses were then treated with 25 μg/mL of emetine or vehicle (water) for 15 minutes at 37°C, and washed 2x with 1X PBS. $5x10^5$ cells or corpses were injected into each rear footpad of naïve C57Bl/6 mice. 24 hours later, animals were sacrificed and the draining (popliteal) lymph nodes were harvested; single cell suspensions were made and stained for flow cytometric analysis.

For cross-priming assays: NIH-3T3 cells expressing membrane-bound ovalbumin (mOVA) were treated with 500 nM AP1 for 3 hours or pulsed for 15 minutes at 37°C as previously described (*14*). Corpses were then treated with 25 μg/mL or emetine or vehicle (water) for 15 minutes at 37°C, and washed 2x with 1X PBS. One million cells were injected into the flank of naïve C57Bl/6 female mice, and nine days later, animals were sacrificed and spleen and draining (inguinal) lymph nodes were harvested and pooled. Single cell suspensions were made and cells were stained for OVA tetramer (SIINFEKL-H2Kb), and activation markers; cells were fixed and analyzed by flow cytometry.

Flow cytometric analysis

Cell viability was assessed by staining with fixable Live/Dead Zombie Green (BioLegend), diluted 1:1000 in PBS and incubated at 4°C for 30 minutes. For surface staining, cells were first incubated with anti-CD16/CD32 receptor antibody (BD clone 2.4G2) diluted 1:100 in PBS, for 30 minutes at 4°C. Cells were then stained with fluorescently conjugated antibodies diluted 1:100 in PBS for 1 hour at 4°C; cell suspensions were stained with antibodies to CD3 (BD clone 145-2C11), B220 (BD clone RA3-6B2), CD4 (BD clone RM4-5), CD8a (BD clone 53-6.7), CD11b (BD clone M1/70), CD11c (BD clone N418), MHCII I-A/E (BD clone M5/114.15.2), F4/80 (BD clone T45-2342), and SIINFEKL-H2Kb tetramer (NIH Tetramer Core).

For intracellular staining, cells were fixed with BD Perm/Fix for 20 minutes at 4°C, and then permeabilized with BD Perm/Wash for 10 minutes at 4°C. Cells were stained intracellularly with fluorescently conjugated antibodies to CCL2 (BD clone 2H5) and IL-6 (BD clone MP5-20F3), diluted 1:100 in BD Perm/Wash, for 1 hour at 4°C.

All flow cytometry was performed on a BD LSRII flow cytometer. FlowJo (Tree Star, Inc.) software was used for all flow cytometric analysis.

Statistics

Statistical analysis was performed with GraphPad Prism software (La Jolla, CA). For *in vivo* experiments, unpaired t test (for percentage) or Mann-Whitney test (for total cell numbers) were used. For ELISA, unpaired t tests were used.

4.5 SUPPLEMENTARY INFORMATION

Figure S1

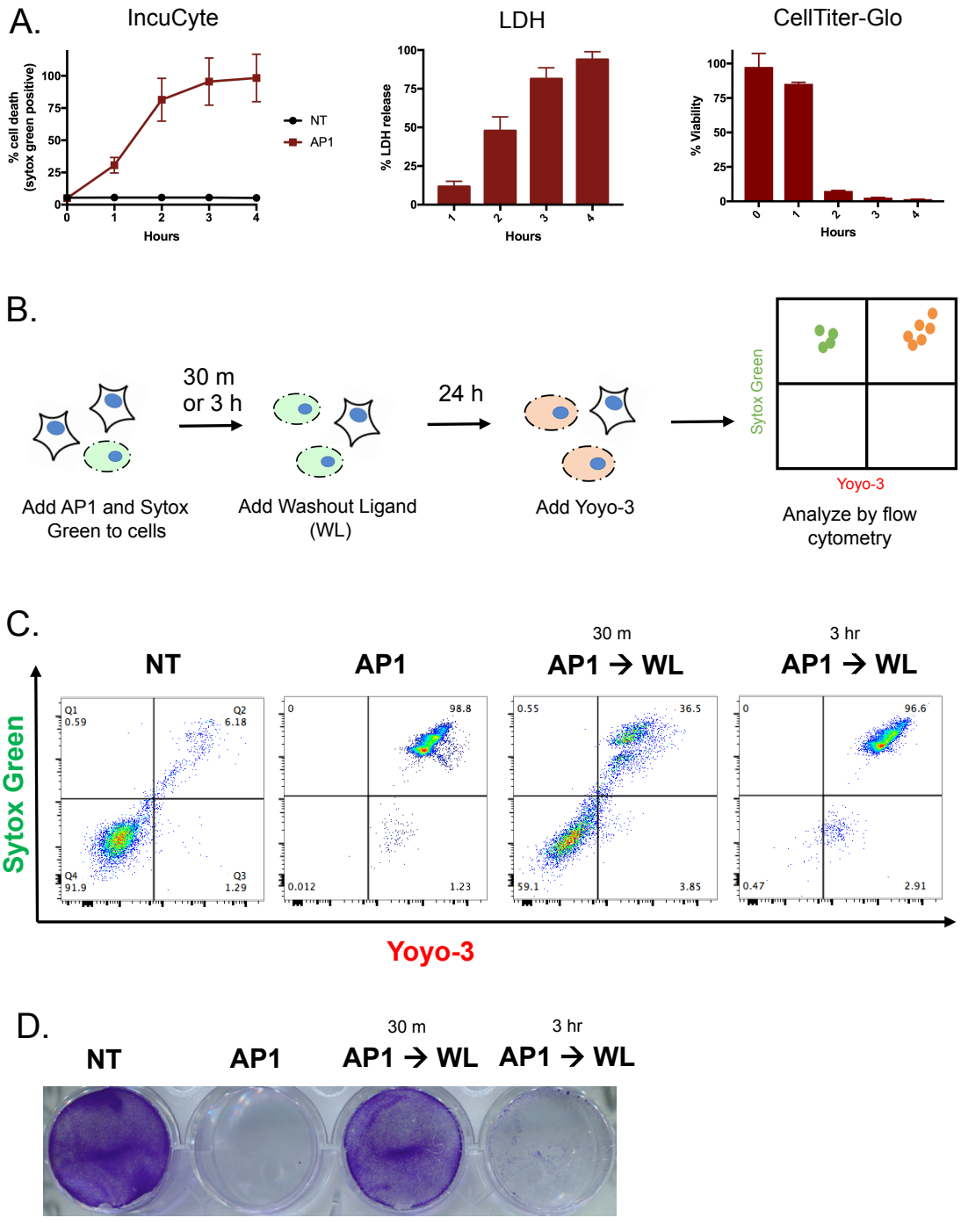


Figure 4.7. Orozco *et al.* Supplementary Figure 1.

Necroptotic NIH-3T3 cells do not recover viability. (A) NIH-3T3 cells expressing RIPK3-2xFV were treated with AP1 to induce necroptosis, and cell death kinetics were analyzed using IncuCyte bioimaging, LDH release, or ATP concentrations. (B) Experimental schematic for (C). (C) NIH-3T3 were treated as in (B); cells were treated with AP1 in the presence of Sytox Green, and Washout Ligand was added at 30 minutes or 3 hours post-AP1 addition. 24 hours later, the red cell-impermeable dye Yoyo-3 was added, and cells were then analyzed by flow cytometry. Representative flow plots from each condition are shown. (D) NIH-3T3 cells were treated with AP1 to trigger necroptosis, +/- Washout Ligand (WL) after 30 minutes or 3 hours. Cells were allowed to recover for 24 hours prior to fixation and staining with crystal violet. All data are representative of at least two independent experiments.

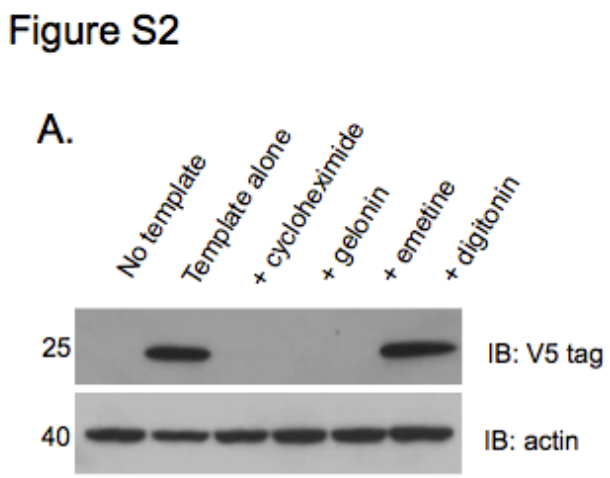


Figure 4.8. Orozco *et al.* Supplementary Figure 2.
In vitro translation assay. (A) The effects of various compounds on translation were assessed in a cell-free translation system, using a construct (template) coding for a V5-tagged protein. Protein levels were analyzed via Western blot analysis. Data are representative of two independent experiments.

Figure S3

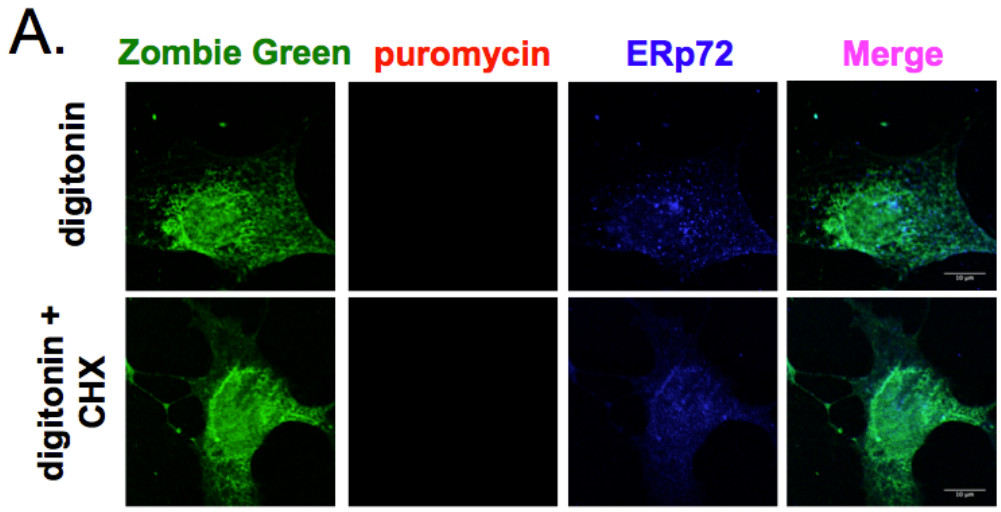


Figure 4.9. Orozco *et al.* Supplementary Figure 3.
Digitonin-permeabilized cells are unable to continue translation. (A) NIH-3T3 cells were permeabilized with the detergent digitonin, followed by a Zombie Green pulse, and then a 10-minute incubation with puromycin +/- cycloheximide (as illustrated in fig. 3A). Cells were then fixed, stained with indicated antibodies, and analyzed via microscopy. Representative images from each condition are shown. Scale bar = 10 μm. Data are representative of two independent experiments.

Figure S4

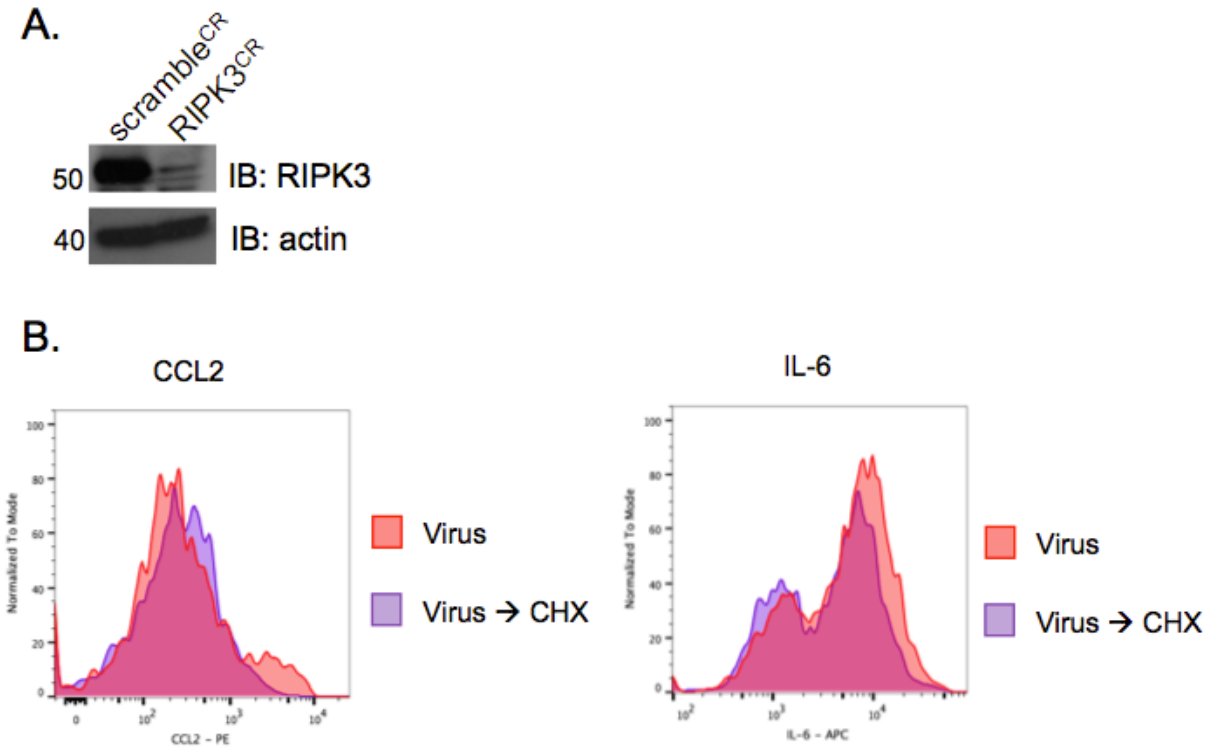


Figure 4.10. Orozco *et al.* Supplementary Figure 4.
MCMV M45ΔRHIM induces RIPK3-dependent death and triggers zombie cytokine translation. (A) Western blot analysis of lysates from SVEC4-10 cells with (scramble^{CR}) or without (RIPK3^{CR}) RIPK3. (B) Representative histograms of cytokines (CCL2, left; IL-6, right) in Zombie Green-positive SVEC4-10 cells that were infected with MCVM M45ΔRHIM at an MOI of 1 for 6 hours. Data in (B) are representative of two independent experiments.

Figure S5

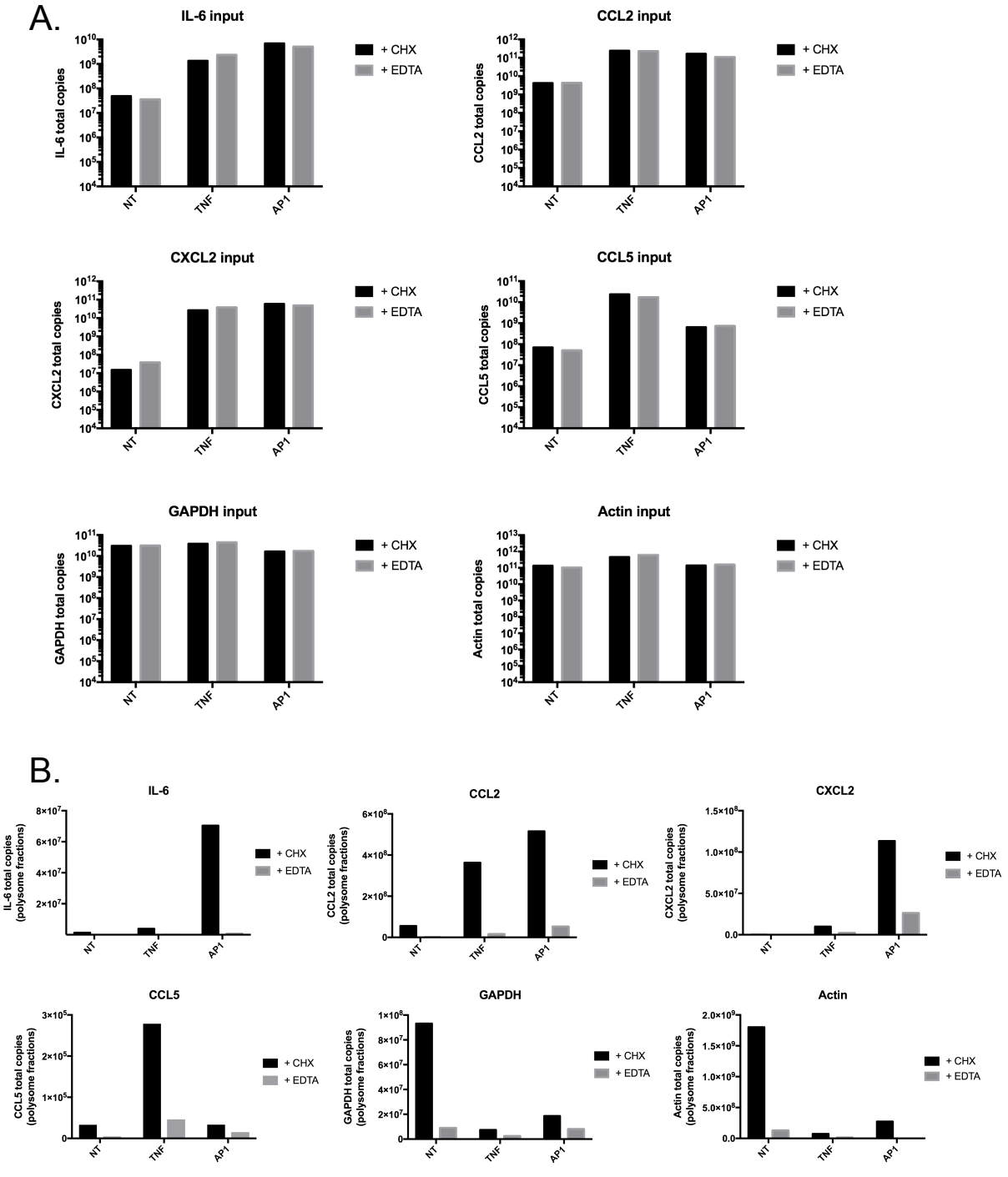


Figure 4.11. Orozco *et al.* Supplementary Figure 5.

Necroptotic zombie cells continue translation of cytokines and chemokines. (A and B) NIH-3T3 cells were treated with either vehicle (No Treatment, “NT”), TNFα, or AP1 for 4 hours, then treated with either CHX or EDTA for 5 minutes. mRNA transcripts in total input cell lysates (A) or polysome fractions (B) were quantified by qPCR. All data are representative of two independent experiments.

Figure S6

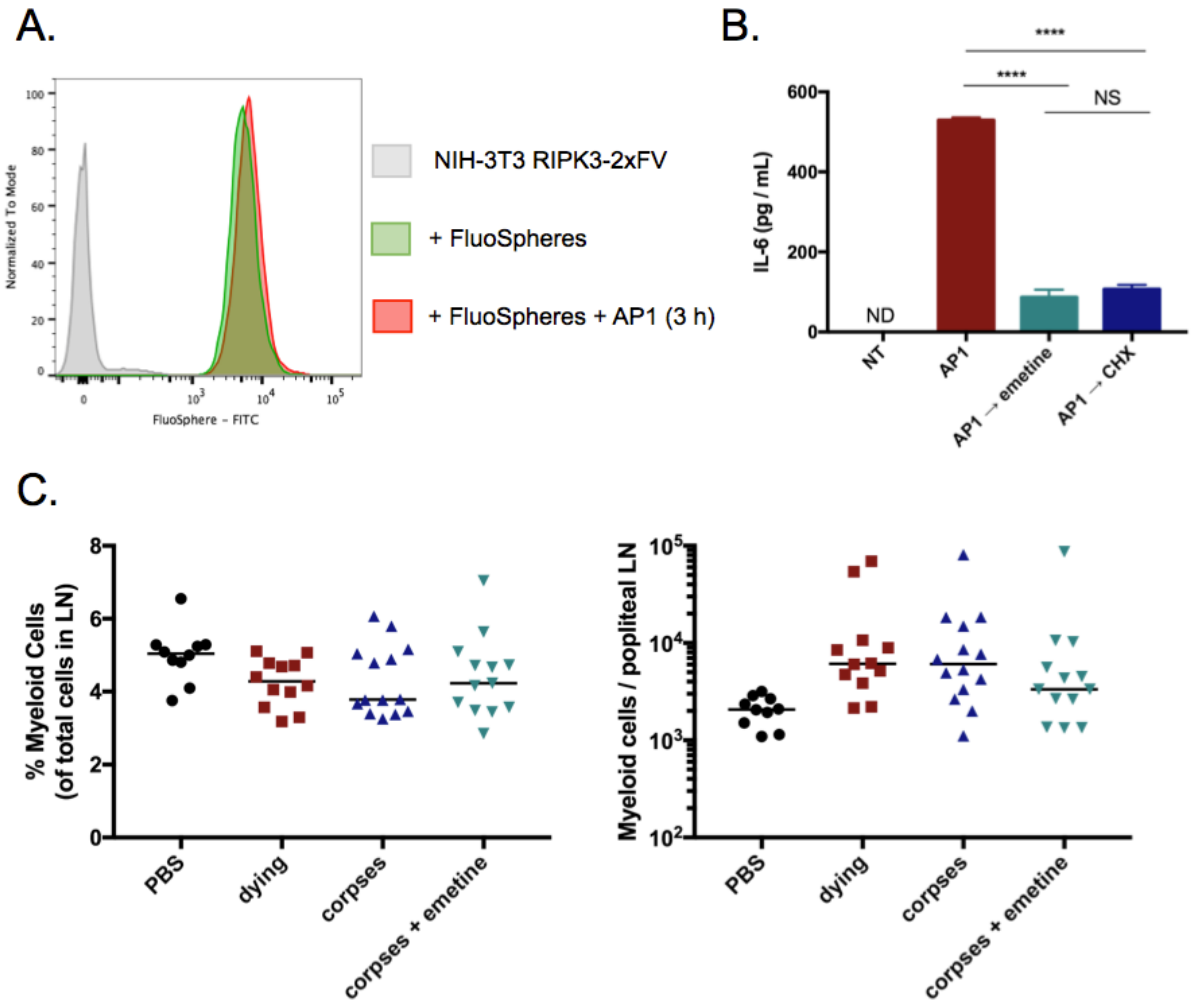


Figure 4.12. Orozco *et al.* Supplementary Figure 6.
Zombie translation influences myeloid cell trafficking. (A) Green-fluorescent FluoSphere nanoparticles were fed to NIH-3T3 cells, then cells were induced to undergo necroptosis for 3 hours and analyzed by flow cytometry. (B) NIH-3T3 cells were treated with AP1 to induce necroptosis, and then treated with either emetine or cycloheximide at 3 hours post-AP1 addition. ELISA analysis was used to quantify IL-6 concentrations in cell culture supernatants at 9 hours post-AP1. (C) Cells were treated as in fig. 6A, and injected into naïve mice. 24 hours later, the animals were sacrificed, draining lymph nodes harvested, and flow cytometric analysis performed. Gating: all cells > singlets > $CD3^{-}B220^{-}CD11b^{+}$.

4.6 ACKNOWLEDGMENTS AND FUNDING

Acknowledgments: We thank Drs. Ram Savan and Lomon So for helpful discussions and technical assistance with polysome experiments; Dr. William Kaiser for generously providing MCMV M45ΔRHIM virus; Dr. Jennifer Martinez for reagents. **Funding:** This work was supported by NIH grants R01AI132595 and R01AI108685 (to A.O.), and NIH NIAID R01 Diversity Supplement (to S.L.O.). **Contributions:** S.L.O., N.Y., M.L.A, and A.O. conceived the project and planned experiments. S.L.O. and B.P.D performed microscopy experiments; S.L.O., M.N.M., and A.G.S. performed *in vivo* experiments; P.J. and A.O. performed cloning; S.L.O. performed cell culture and related experiments; S.L.O. performed polysome experiments. N.Y., M.L.A., and S.W.G.T. provided reagents. B.P.D., M.N.M., N.Y., A.G.S., S.W.G.T. and M.L.A. contributed insightful discussions. S.L.O and A.O. wrote the manuscript, and all authors contributed to its revision. **Competing interests:** All authors declare no competing interests. **Data and materials availability:** All data is available in the main text or the supplementary materials.

4.7 REFERENCES

1. N. Yatim, S. Cullen, M. L. Albert, Dying cells actively regulate adaptive immune responses. *Nature reviews. Immunology* **17**, 262-275 (2017).
2. D. R. Green, F. Llambi, Cell Death Signaling. *Cold Spring Harb Perspect Biol* **7**, (2015).
3. N. M. de Vasconcelos, N. Van Opdenbosch, M. Lamkanfi, Inflammasomes as polyvalent cell death platforms. *Cell Mol Life Sci* **73**, 2335-2347 (2016).
4. L. Vande Walle, M. Lamkanfi, Pyroptosis. *Curr Biol* **26**, R568-572 (2016).

5. A. Degterev *et al.*, Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol* **4**, 313-321 (2008).

6. Y. Lin *et al.*, Tumor necrosis factor-induced nonapoptotic cell death requires receptor-interacting protein-mediated cellular reactive oxygen species accumulation. *The Journal of biological chemistry* **279**, 10822-10828 (2004).

7. Y. S. Cho *et al.*, Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* **137**, 1112-1123 (2009).

8. D. W. Zhang *et al.*, RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* **325**, 332-336 (2009).

9. S. He *et al.*, Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* **137**, 1100-1111 (2009).

10. W. Chen *et al.*, Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling. *The Journal of biological chemistry* **288**, 16247-16261 (2013).

11. J. Wu *et al.*, Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. *Cell Res* **23**, 994-1006 (2013).

12. L. Sun *et al.*, Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* **148**, 213-227 (2012).

13. J. Zhao *et al.*, Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 5322-5327 (2012).

14. N. Yatim *et al.*, RIPK1 and NF-kappaB signaling in dying cells determines cross-priming of CD8(+) T cells. *Science* **350**, 328-334 (2015).

15. S. Orozco *et al.*, RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. *Cell death and differentiation* **21**, 1511-1521 (2014).

16. Y. N. Gong *et al.*, ESCRT-III Acts Downstream of MLKL to Regulate Necroptotic Cell Death and Its Consequences. *Cell* **169**, 286-300 e216 (2017).

17. E. K. Schmidt, G. Clavarino, M. Ceppi, P. Pierre, SUnSET, a nonradioactive method to monitor protein synthesis. *Nat Methods* **6**, 275-277 (2009).

18. Z. Cai *et al.*, Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. *Nat Cell Biol* **16**, 55-65 (2014).

19. G. Quarato *et al.*, Sequential Engagement of Distinct MLKL Phosphatidylinositol-Binding Sites Executes Necroptosis. *Mol Cell* **61**, 589-601 (2016).

20. Y. Dondelinger *et al.*, MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. *Cell Rep* **7**, 971-981 (2014).

21. N. S. Wang, M. T. Unkila, E. Z. Reineks, C. W. Distelhorst, Transient expression of wild-type or mitochondrially targeted Bcl-2 induces apoptosis, whereas transient expression of endoplasmic reticulum-targeted Bcl-2 is protective against Bax-induced cell death. *The Journal of biological chemistry* **276**, 44117-44128 (2001).

22. J. W. Upton, W. J. Kaiser, E. S. Mocarski, DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. *Cell host & microbe* **11**, 290-297 (2012).

23. R. J. Thapa *et al.*, DAI Senses Influenza A Virus Genomic RNA and Activates RIPK3-Dependent Cell Death. *Cell host & microbe* **20**, 674-681 (2016).

24. T. Kuriakose *et al.*, ZBP1/DAI is an innate sensor of influenza virus triggering the NLRP3 inflammasome and programmed cell death pathways. *Sci Immunol* **1**, (2016).

25. S. Nogusa *et al.*, RIPK3 Activates Parallel Pathways of MLKL-Driven Necroptosis and FADD-Mediated Apoptosis to Protect against Influenza A Virus. *Cell host & microbe* **20**, 13-24 (2016).

26. H. Chasse, S. Boulben, V. Costache, P. Cormier, J. Morales, Analysis of translation using polysome profiling. *Nucleic Acids Res* **45**, e15 (2017).

27. A. P. Grollman, Inhibitors of protein biosynthesis. V. Effects of emetine on protein and nucleic acid biosynthesis in HeLa cells. *The Journal of biological chemistry* **243**, 4089-4094 (1968).

28. M. J. Clemens, M. Bushell, I. W. Jeffrey, V. M. Pain, S. J. Morley, Translation initiation factor modifications and the regulation of protein synthesis in apoptotic cells. *Cell death and differentiation* **7**, 603-615 (2000).

29. X. Saelens *et al.*, Protein synthesis persists during necrotic cell death. *J Cell Biol* **168**, 545-551 (2005).

30. H. Wang *et al.*, Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. *Mol Cell* **54**, 133-146 (2014).

31. C. J. Kearney *et al.*, Necroptosis suppresses inflammation via termination of TNF- or LPS-induced cytokine and chemokine production. *Cell death and differentiation* **22**, 1313-1327 (2015).

32. E. E. Gray *et al.*, The AIM2-like Receptors Are Dispensable for the Interferon Response to Intracellular DNA. *Immunity* **45**, 255-266 (2016).

33. N. E. Sanjana, O. Shalem, F. Zhang, Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* **11**, 783-784 (2014).

34. B. P. Daniels *et al.*, RIPK3 Restricts Viral Pathogenesis via Cell Death-Independent Neuroinflammation. *Cell* **169**, 301-313 e311 (2017).

Chapter 5. CONCLUSIONS

5.1 SUMMARY OF FINDINGS

Here we described a system of activatable RIPK3, which we used to explore both mechanisms of RIPK3 activation and necroptosis, as well as the immunological outcomes of necroptotic death. We first showed that RIPK1 can both activate and inhibit RIPK3 oligomerization and necroptosis, and RIPK1's inhibitory function was independent of its kinase activity (*1*). We then used this system to explore how antigen derived from dying cells is cross-presented to CD8+ T cells, and found a requirement for RIPK1 and NF-κB activation to get efficient cross-priming of necroptotic cell-derived antigen (*2*). Finally, we found that cells that undergo necroptosis, and are considered "dead" by many commonly used assays, can continue producing pro-inflammatory mediators; these "corpses" can continue the translation of cytokines and chemokines, and we termed this phenomenon "zombie translation." We found that zombie translation of these pro-inflammatory molecules was necessary for efficient uptake and migration of innate immune cells to draining lymph nodes, as well as in cross-priming of CD8+ T cell responses.

5.2 ONGOING QUESTIONS

Although we have begun to uncover some mechanisms of how necroptosis is inflammatory, many questions remain. How different forms of cell death are engaged by pathogens and "seen" by early responders, such as neutrophils, macrophages, and dendritic cells, is still an open question. Is a cell that dies by apoptosis from influenza infection less immunogenic than a cell that dies by necroptosis?

Furthermore, because RIPK3 activation results in the coordinated process of pro-inflammatory molecule production, as well as cell death, it is difficult to tease apart the contribution of each component to setting up an efficient immune response. Though we began to address the contribution of NF-κB activation versus cell death by using the RIPK3ΔC-2xFV construct, we showed that this particular version of RIPK3 induces the upregulation of several genes that are RIPK3 kinase-dependent but RHIM-independent. How RIPK3 kinase activity alone can lead to transcription (and perhaps translation?) of these genes is a completely black box.

Similarly, we now appreciate that RIPK1 is necessary for efficient induction of NF-κB activity downstream of RIPK3 activation (*2*), but the mechanism for this is very unclear. How do caspase-8 and cFLIP contribute to this transcriptional program? Caspase-8 has been shown to have transcriptional roles in other settings (*3, 4*), so it is tempting to speculate that caspase-8 may have similar functions downstream of RIPK3 activation.

Furthermore, although we identified zombie translation as necessary for shaping immune responses to necroptotic-cell derived antigen, it is still unclear when (if at all) this phenomenon would happen *in vivo*. Dying cells are usually cleared rapidly and efficiently by phagocytes, so the question remains: when would a necroptotic cell persist *in vivo* long enough to lose cell membrane integrity and continue translation? Our data would hint that zombie translation may happen *in vivo* in response to a viral infection, but this has not been directly tested.

In addition, other general questions around RIPK3 and necroptosis remain. What is the contribution of necroptosis (and specifically, RIPK3 activity) to human disease? As mentioned in chapter 2, RIPK3 has been implicated as deleterious in a number of disease models in mice, but how well does this translate to humans? Further, several small molecule compounds have been

developed and shown to inhibit RIPK3 kinase activity (*5, 6*). While these RIPK3 inhibitors could have therapeutic potential, studies have shown that these small molecules can induce apoptosis *in vitro (5)*. Context is important, and further studies will be necessary to determine when RIPK3 activity is helpful or detrimental.

5.3 REFERENCES

1. S. Orozco *et al.*, RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. *Cell death and differentiation* **21**, 1511-1521 (2014).
2. N. Yatim *et al.*, RIPK1 and NF-kappaB signaling in dying cells determines cross-priming of CD8(+) T cells. *Science* **350**, 328-334 (2015).
3. N. H. Philip *et al.*, Activity of Uncleaved Caspase-8 Controls Anti-bacterial Immune Defense and TLR-Induced Cytokine Production Independent of Cell Death. *PLoS Pathog* **12**, e1005910 (2016).
4. C. M. Henry, S. J. Martin, Caspase-8 Acts in a Non-enzymatic Role as a Scaffold for Assembly of a Pro-inflammatory "FADDosome" Complex upon TRAIL Stimulation. *Mol Cell* **65**, 715-729 e715 (2017).
5. P. Mandal *et al.*, RIP3 induces apoptosis independent of pronecrotic kinase activity. *Mol Cell* **56**, 481-495 (2014).
6. W. J. Kaiser *et al.*, Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. *The Journal of biological chemistry* **288**, 31268-31279 (2013).

APPENDIX A. LIST OF PUBLISHED ARTICLES

Susana Orozco, Nader Yatim, Margo R. Werner, Huey Tran, Sushma Yechan Gunja, Stephen W.G. Tait, Matthew L. Albert, Douglas R. Green, and Andrew Oberst. RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. *Cell Death Differ.* 2014 Oct;21(10):1511-21. PMID: 2490290. https://www.ncbi.nlm.nih.gov/pubmed/24902904

Nader Yatim, Hélène Jusforgues-Saklani, **Susana Orozco**, Oliver Schulz, Rosa Barreira da Silva, Caetano Reos e Sousa, Douglas R. Green, Andrew Oberst, and Matthew L. Albert. RIPK1 and NF-kB signaling in dying cells determines cross-priming of CD8+ T cells. *Science.* 2015 Oct;350(6258):328-34. PMID: 26405229. https://www.ncbi.nlm.nih.gov/pubmed/26405229

Brian P. Daniels, Annelise G. Snyder, Tayla M. Olsen, **Susana Orozco**, Thomas H. Oguin III, Stephen W.G. Tait, Jennifer Martinez, Michael Gale, Jr., Yueh-Ming Loo, and Andrew Oberst. RIPK3 Restricts Viral Pathogenesis via Cell-Death Independent Neuroinflammation. *Cell.* 2017 Apr 6;169(2):301-313.e11. PMID: 28366204. https://www.ncbi.nlm.nih.gov/pubmed/28366204

Susana Orozco and Andrew Oberst. RIPK3 in cell death and inflammation: the good, the bad, and the ugly. *Immunol Rev.* 2017 May;277(1):102-112. PMID: 28462521. https://www.ncbi.nlm.nih.gov/pubmed/28462521

Evangelos Giampazolias, Barbara Zunino, Sandeep Dhayade, Florian Bock, Catherine Cloix, Kai Cao, Alba Roca, Jonathan Lopez, Gabriel Ichim, Emma Proïcs, Camila Rubio-Patiño, Loic Fort, Nader Yatim, Emma Woodham, **Susana Orozco**, Lucia Taraborrelli, Nieves Peltzer, Daniele Lecis, Laura Machesky, Henning Walczak, Matthew L. Albert, Simon Milling, Andrew Oberst, Jean-Ehrland Ricci, Kevin M. Ryan, Karen Blyth and Stephen W. G. Tait. Mitochondrial permeabilization engages NF−κB-dependent anti-tumour activity under caspase deficiency. *Nat Cell Biol.* 2017 Sep;19(9):1116-1129. PMID: 28846096. https://www.ncbi.nlm.nih.gov/pubmed/28846096

Susana L. Orozco, Brian P. Daniels, Nader Yatim, Michelle N. Messmer, Annelise G. Snyder, Pooja Jain, Stephen W.G. Tait, Matthew L. Albert, and Andrew Oberst. RIPK3 activation leads to continued cytokine synthesis after loss of cell membrane integrity. *(Manuscript submitted)*

VITA

Susana Orozco grew up part-time in San Miguel de Allende, Guanajuato, Mexico and San Diego, CA. She attended the University of California, Berkeley for her undergraduate work, and graduated with her Bachelor of Arts in Molecular and Cell Biology in May 2007. After graduation, she taught middle school science in Oakland, CA at Edna Brewer Middle School as part of Teach For America (TFA). Susana concurrently earned her Masters of Arts in Education at Alliant International University, and graduated in December 2009. Thereafter, she worked as a research assistant in the laboratory of Dr. Eva Harris, helping develop a mouse-adapted strain of dengue virus to be used in an animal model. It was during this time that Susana fell in love with research, and she joined the Molecular and Cellular Biology Graduate Program at the University of Washington in the fall of 2012. In addition to being a science nerd, Susana enjoys attending Seattle Sounders FC matches, delving into the world of wine and whiskey, engaging in creative writing and painting, as well as cooking and occasionally doing exercise. Upon conclusion of her graduate studies, Susana will be pursuing an academic postdoctoral position, so she can continue doing research.