Engagement of programmed cell death by nucleic acid sensing

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Abstract

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During infection, the sensing of foreign nucleic acids inside an infected cell is often the first line of immunological defense. When DNA or RNA is sensed, one outcome is the production of anti-viral signaling molecules such as type I interferons. Additionally, cells can undergo programmed cell death. Here we describe the mechanism of induction of the programmed cell death pathway, necroptosis, following detection of cytosolic DNA or RNA. Necroptosis is a lytic and proinflammatory form of cell death, that can eliminate infected cells and alert and instruct the immune system. We show that following sensing of foreign nucleic acids, induction of necroptosis relies on the production of both anti-viral interferon, as well pro-inflammatory molecule, tumor necrosis factor.

Table of contents

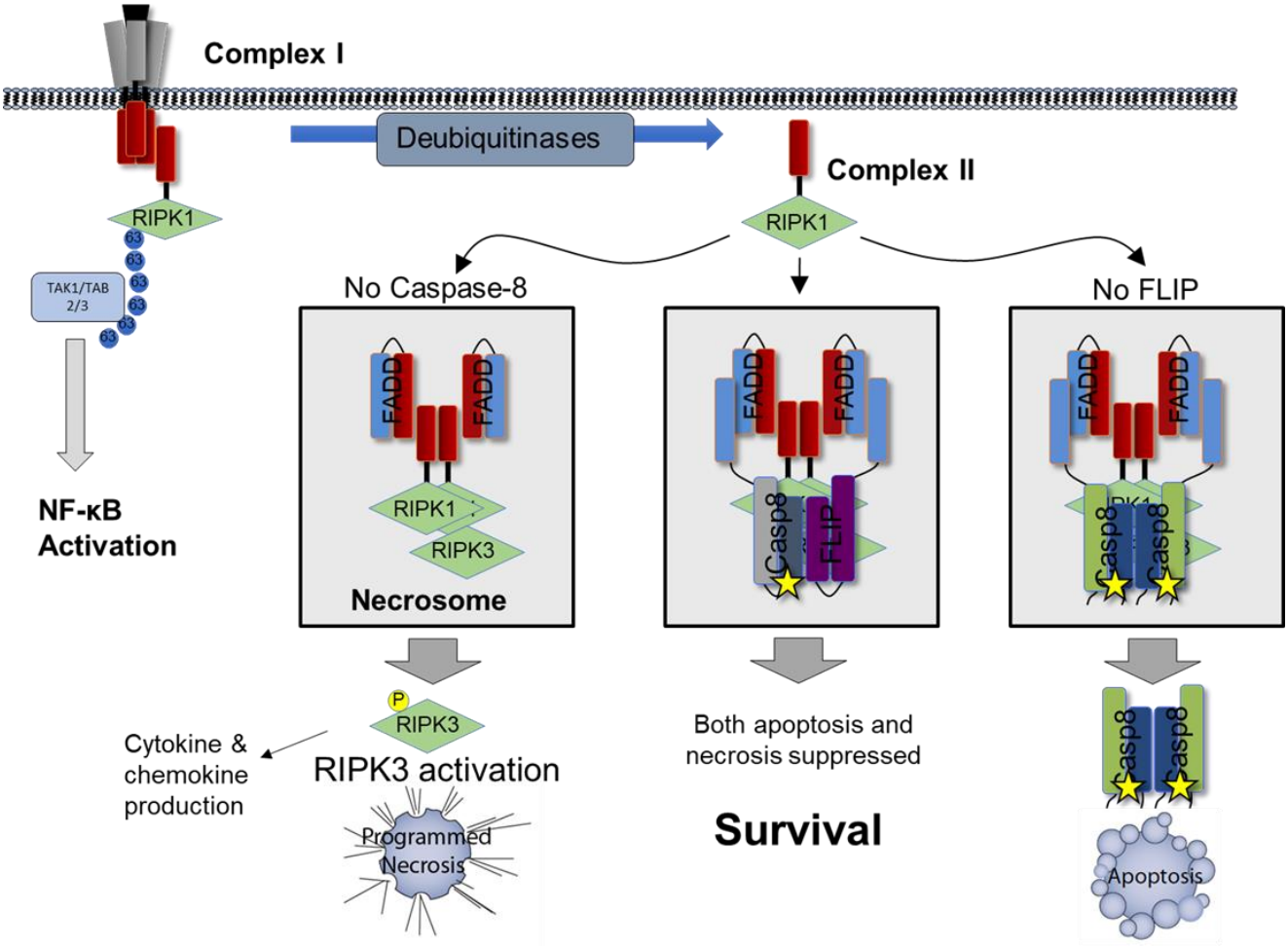
Chapter 1: Introduction

Inflammation and necroptosis

Inflammation is a critical step in responding to infection, wounds, and other disturbances to homeostatic conditions. One important mediator of inflammation is tumor necrosis factor, or TNF. TNF is canonically thought to be a proinflammatory survival signal, despite its discovery as an inducer of tumor cytotoxicity[1]. It is produced downstream of numerous signaling pathways, and has pleotropic effects. However, as its name suggests, this pro-survival signal has been shown to be entangled in numerous cell death pathways (Figure 1). Canonically, TNF signaling occurs at the cell membrane when the TNF receptor 1 (TNFR1) sees its ligand, TNF[2]. Receptor ligation induces the assembly of a complex, known as complex I, at the membrane, which contains numerous adaptor molecules, such as tumor necrosis factor receptor type 1-associated death domain protein (TRADD) and the TNF receptor associated factors (TRAFs), along with receptor-interacting protein kinase 1 (RIPK1), and the cellular inhibitors of apoptosis (cIAPs). Complex I can signal through MAPKS, IKK proteins, and NF-κB[3] to upregulate numerous proinflammatory cytokines and chemokines, as well as pro-survival molecules. However, multiple changes in the cellular context can allow for transition of complex I from the membrane into the cytosol, where it is known as complex II. This transition can occur when proper ubiquitination of complex I is disturbed, or if cIAPs are degraded[4,5]. Complex II associates with the adaptor molecule Fas-associated protein with death domain (FADD), which recruits a member of the cysteine-aspartic proteases (caspases), caspase-8, to the complex[6]. When caspase-8

becomes activated in this complex, it initiates apoptotic cell death. Pro-survival signals such as the upregulation of caspase-8's binding partner, cellular FLICE-inhibitory protein (cFLIP), keep the initiation of apoptosis in check[7]. However, apoptosis is not the only cell death outcome of TNF signaling. If caspase-8 or cFLIP is not functional, absent, or somehow blocked from entering complex II, a complex known as the necrosome can form[8]. Necrosome formation leads to the oligomerization of RIPK3, its activation, and subsequently death of the cell by necroptosis, or programmed necrosis[9].

Figure 1 – Death receptor signaling outcomes depend on cellular state



Necroptosis is a form of cell death that is mechanistically distinct from other forms of programmed cell death. While both apoptosis and pyroptosis (discussed in detail later) require caspase activation for death to occur, necroptosis requires the RIP kinases[10,11,12], and often the suppression of caspases. In this way, it is thought to be an emergency form of cell death that may have evolved to kill cells when apoptosis is defective or blocked. Much of what we know about necroptosis comes from studying its activation downstream of TNF signaling and caspase suppression, though many signals can activate the RIP kinases. All known forms of necroptosis activate RIPK3, which in turn phosphorylates and activates the protein mixed lineage kinase domain-like (MLKL), the executioner of necroptosis. Thus, while many signals can induce this outcome, once RIPK3 is activated, the cell death process looks largely the same. (Of note, RIPK3 activation does not always lead to the outcome of cell death, more on this later.) When RIPK3 is activated to kill the cell, it phosphorylates MLKL, which oligomerizes and associates with the plasma membrane[13,14]. MLKL contains an N-terminal helical bundle that upon activation, acts similarly to a pore-forming toxin and punches holes in the cell membrane, leading to cellular swelling and rupture. Due to the lytic nature of this cell death pathway, it is thought to be much more inflammatory than apoptosis, where the cell shrinks and forms apoptotic bodies for phagocytes to clean up. Additionally, it has been more recently appreciated that this cell death is accompanied by a burst of cytokines that can attract and instruct nearby immune cells[15].

As the discovery of RIPK3 and necroptosis was studied mostly in the context of TNF signaling, much of the mechanism of activation is known in that context. When TNF signaling is active and caspase 8 is suppressed or absent, cFLIP can no longer

play a pro-survival role (Figure 1). RIPK1 becomes activated, and phosphorylates RIPK3. This activation event leads to the oligomerization of RIPK3, and its subsequent activation of MLKL. Interestingly, RIPK1 can also play an inhibitory role[16]. By interacting with both RIPK3 and FADD/caspase-8, RIPK1 bridges this inhibitory complex to RIPK3. However, when caspase-8 is inhibited or absent, RIPK1 is required to activate RIPK3.

RIPK1 interacts with RIPK3 through their shared RIP homotypic interaction motifs, or RHIM domains[17]. Only four proteins in the mammalian genome contain RHIM domains – RIPK1, RIPK3, TIR-domain-containing adapter-inducing interferon-β (TRIF)[18], and DNA-dependent activator of IFN-regulatory factors (DAI)[19]. To date, all known mechanisms of RIPK3 activation work through direct RHIM-RHIM interactions with one of these other molecules. Toll-like receptor 3 (TLR3) and TLR4, two innate pattern recognition receptors, signal through the adapter molecule TRIF, which interacts directly with RIPK3[20], leading to cell death downstream of both double-stranded RNA (dsRNA) or lipopolysaccharide (LPS) detection, respectively.

RIPK3 is required for host survival during numerous viral infections – vaccinia virus (VacV)[21], herpes simplex virus (HSV)[22], influenza virus (IAV)[23], and West Nile virus (WNV)[24], to name a few. The fact that *Ripk3*$^{-/-}$ mice lack an overt phenotype until infected with one of these viruses implies that the most important function of RIPK3 is its antiviral role. However, for many viral infections it is unclear how RIPK3 becomes activated. In the case of RNA viruses, TLR3 has been suggested as a potential sensor, as this signaling pathway was already well described to activate RIPK3. However, for DNA viruses, the data were less clear. While it has been shown that vaccinia virus requires DAI for RIPK3 activation[21], it was unclear if this activation was from the viral

DNA itself, or from some other part of the viral life cycle. More recently, it has been shown that DAI is required to induce necroptosis following influenza infection[25,26]; as influenza virus has an RNA genome, this result challenges the notion that DAI is a bona fide DNA sensor.

Furthermore, other innate immune responses to these viruses require intracellular RNA or DNA sensing through the mitochondrial antiviral-signaling protein (MAVS) or stimulator of interferon genes (STING) pathways, particularly when it comes to the production of type I interferon (IFN), a critical molecule in antiviral defense. Intriguingly, type I IFN was recently discovered to play an important role in the activation and execution of necroptosis[27]. However, it remained unclear how IFN was activating RIPK3, as no conical pathway seemed to be playing a role. One paper claimed that RIPK3 was activated by phosphorylation by protein kinase R (PKR)[28], an RNA sensor and IFN-stimulated gene (ISG); however this would be the first PKR target outside of its canonical function, and the only way to activate RIPK3 without direct RHIM-RHIM interaction. It was also unclear how PKR itself would become activated in the absence of dsRNA, its normal ligand. PKR self-activation by upregulation alone would seemingly be a costly plan for the cell if the outcome of this activation is cell death. However, it was very clear that type I IFN was playing a critical role in either the regulation or activation of programmed necrosis.

Type I Interferon and nucleic acid sensing

Type I IFN sets off a cascade of transcriptional changes that causes the upregulation of thousands of gene targets. This upregulation event is critical for the

establishment of the “antiviral state,” which protects animals from numerous viral infections[29]. Some of these upregulated targets directly interact and restrict the viral life cycle. For example, the RNA sensor mentioned earlier, PKR, is upregulated upon stimulation by IFN, and becomes activated when foreign RNA is detected. Once activated, PKR shuts down translation through inactivation of eIF2α[30]. Without the ability to translate its gene products, the virus is stopped in its tracks. Another ISG, tetherin, acts farther downstream the viral life cycle. Viruses must either bud from the cell surface, or lyse the cell to escape into the extracellular space and infect new cells or hosts. Tetherin, as its name might imply, acts as a tether for budding viruses[31], inhibiting viral escape and ability to infect new cells. In these ways and many others[32], IFN plays a critical role in the defense against viruses. Mice lacking the IFN-alpha/beta receptor (IFNAR) or interferon-related transcription factors are exquisitely sensitive to numerous RNA and DNA viruses[33]. As RIPK3-deficient mice also display susceptibility to many of these viruses, and IFN was shown to play an important role in allowing necroptosis to occur, it is tempting to hypothesize that one reason IFNAR-deficient mice are susceptible is due to defective necroptosis, among other defects.

Interferon can be produced through TLR signaling, as well as downstream of intracellular nucleic acid sensing. When a virus infects a cell, often the fastest way for the cell to detect its presence is by the introduction and sensing of foreign viral genomes. For this reason, nucleic acid sensing is an important part of the innate immune response to infection. Nucleic acid sensing pathways come in two major flavors: RNA and DNA. The intracellular RNA sensing pathways that lead to the upregulation of IFN have multiple sensors that converge on the adaptor MAVS[34].

Meanwhile, the IFN-producing intracellular DNA sensing pathway requires the activation of the sensor cyclic GMP-AMP synthase (cGAS)[35,36] and adaptor STING[37,38]. These pathways look largely similar after their initial sensing events: the adaptor molecule (either MAVS or STING) recruits TANK-binding kinase 1 (TBK-1) and interferon regulatory factor (IRF3), and activates these molecules, leading to the upregulation and production of IFN-β. Without these sensing events, hosts cannot mount a proper innate immune response to infection.

The AIM2 inflammasome and pyroptosis

DNA sensing can be carried out by multiple sensors. While cGAS triggers STING-dependent IFN production, another DNA sensor, absent in melanoma 2 (AIM2), does not trigger the production of IFN. Rather, AIM2 activation triggers formation of a large molecular complex in the cell, called the inflammasome[39]. Inflammasomes contain three main proteins: a sensor of some type (in this case, AIM2), the adaptor apoptosis-associated speck-like protein containing a CARD (ASC), and the protease caspase-1[40]. Inflammasome assembly activates caspase-1, which among other targets, can cleave pro-IL-1β and pro-IL-18 into their biologically active forms[41]. There are many signals that can activate caspase-1, not just AIM2. NLRP3, a member of the NOD-like receptor family, is a well-studied caspase-1 activator that senses many perturbations to the cellular environment, such as ion flux and crystalline structures[42]. Additionally, NLRC4 detects bacterial flagellin[43]. These sensors and many others work to defend against infection and cellular perturbation and allow for induction of pro-inflammatory signals in response.

In addition to pro-inflammatory cytokines, caspase-1 (and related caspase-11) can cleave another target – gasdermin-D (GSDMD)[44]. Recently described, GSDMD acts similarly to MLKL and pokes holes in the membranes of the cell[45], leading to cellular rupture and death, and elimination of perturbed or infected cells. This caspase-1 dependent cell death is called pyroptosis, and occurs in cells of the myeloid lineage. More recently, it has been appreciated that a select few other cell types can undergo this form of cell death, such as epithelial cells in the gastrointestinal tract[46]. However, in many cell types, inflammasome signaling and pyroptosis does not occur, and the response to foreign DNA is driven by the cGAS-STING pathway, producing IFNβ. While IFN production is critical for many anti-viral processes, it was not clear if infected cells that could not undergo pyroptosis would be eliminated by another programmed cell death pathway. Therefore, we wondered whether DNA could induce programmed necrosis when the pyroptosis pathway was not available, either by direct caspase suppression, or in cell types that lack functional inflammasome pathways. Furthermore, we wondered whether any programmed necrosis downstream of nucleic acid sensing would require IFN production and signaling. These foundational questions led to the research that follows describing the mechanism of DNA and RNA-induced necroptosis.

Chapter 2: Controlled Detonation: Evolution of Necroptosis in Pathogen Defense

Introduction:

The following is an invited review I wrote for the Immunology and Cell Biology journal, published in December 2016. While it does review key findings in the field, it focuses on how those findings might hint at an evolutionary reason or advantage for necroptosis which, of course, cannot be definitively confirmed. Additionally, it identifies many gaps in our understanding of necroptosis and its function, and questions for future research. For this reason, I consider it more of a prospective on the field and ponderings on the real evolutionary reason to develop necroptosis as an innate immune strategy.

Review:

Controlled Detonation: Evolution of Necroptosis in Pathogen Defense

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Abstract:

Necroptosis is a lytic form of programmed cell death that involves the swelling and rupture of dying cells. While several necroptosis-inducing stimuli have been defined, in most cells this pathway is kept in check by the action of the pro-apoptotic protease caspase-8 and the IAP ubiquitin ligases. How and when necroptosis is triggered under

physiological conditions therefore remains a persistent question. Because necroptosis likely arose as a defensive mechanism against viral infection, exploration of this question requires a consideration of host-pathogen interactions, and how the sensing of infection could sensitize cells to necroptosis. Here, we will discuss the role of necroptosis in the response to viral infection, consider why the necroptotic pathway has been favored during evolution, and describe emerging evidence for death-independent functions of key necroptotic signaling components.

Introduction

Programmed cell death is an important part of development, homeostasis, and pathogen defense. As we develop, cell populations expand and contract, forming critical parts of us like the digits of our fingers and the folds of our brains. Once we reach adulthood, billions of our cells are turned over and replaced by others each day, and when we are infected with an invading pathogen, cell death can be an important component of the immune response leading to the clearance of that infection and survival of the host. Most developmental and homeostatic cell death is carried out by the caspase proteases, via the well-described pathway of apoptosis. From the point of view of the immune system, this death is quiet: the apoptotic cell condenses and packages itself for rapid and uneventful elimination by phagocytes.

The mechanism and morphology of apoptosis stand in contrast to that of necroptosis, a more recently described form of programmed cell death. Necroptosis is carried out by the receptor interacting protein kinases, RIPK1 and RIPK3, and their activation results in cellular swelling, bursting, and the release of intracellular contents

into the extracellular space. Interestingly, RIPK3 seems to require the suppression, rather than the activation, of a key apoptotic caspase—caspase-8—to carry out its pro-death role. These characteristics lead to a few interesting questions:

- What benefit is conferred on the host by the existence of the RIP kinase pathway, and of necroptotic cell death?
- Is there an immunological benefit to the lytic, inflammatory nature of necroptosis?
- Why have we evolved a form of programmed cell death that seemingly only occurs when another cell death process cannot be carried out?

These questions are linked; answering the second and third may help us answer the first. The lytic nature of necroptosis would be presumed to stimulate inflammation, and promote immune cell recruitment. This would be undesirable if the point were tissue homeostasis or development, but beneficial during an infection. But why would this occur preferentially when caspases are inhibited? Some pathogens target the caspase-8 apoptotic pathway, possibly to prevent killing of infected cells by Fas ligand on the surface of responding CD8+ T cells. This targeting might explain the emergence of a cell death pathway that is activated only when caspase-8 is blocked but perhaps there are other non-death-related functions of the RIP kinases and caspase-8 have driven the emergence and interplay of the necroptotic and apoptotic pathways.

Before discussing roles for RIPK signaling in the host response to pathogens, let us briefly consider the pathway of necroptosis as it is currently understood. This pathway has been extensively reviewed elsewhere[1-3], so here we will provide only a brief summary. RIPK3 can be activated downstream of several receptors, including TNFR1,

Fas, TRAIL-R, TLR-3 and -4, and by the interferon (IFN)-inducible innate sensor DAI. However, it has been most extensively characterized downstream of TNF signaling. In most cellular contexts, TNF is a pro-survival signal. Upon engagement, its receptor interacts with numerous cytosolic proteins including RIPK1, forming a receptor-associated complex that triggers NF-kB activation[4]. Subsequently, it is thought that RIPK1 can translocate to the cytosol, where it forms additional complexes. One of these is a complex that contains RIPK3[5], the so-called necrosome. Formation of this complex requires the interaction of the RIP homotypic interaction motif (RHIM) domains present in both RIPK1 and RIPK3 and can lead to RIPK3 activation and cell death, but is normally prevented from doing so by the action of a heterodimeric enzyme complex composed of caspase-8 and cFLIP[6] (Fig. 1). These enzymes are recruited to RIPK1 via the adapter FADD and cooperate with E3 ubiquitin ligases, the IAPs, to abrogate necrosome formation and prevent necroptosis[7,8]. Notably, cFLIP is a target of NF-kB, meaning that RIPK1 signaling at the TNFR1 complex leads to up-regulation of a protein that will prevent its participation in necroptotic signaling. This regulation is essential to survival, as genetic deletion of caspase-8 is lethal during embryonic development, but is fully rescued by the additional deletion of RIPK3[9]. Notably then, the necrosome can stably form only when caspase-8 is absent or inhibited, or perhaps when cFLIP upregulation or IAP function is blocked. In such conditions, RIPK1 and RIPK3 can oligomerize and activate MLKL, a pseudokinase that translocates to the plasma membrane, leading to the cellular swelling and rupture that characterizes necroptotic cell death[10-13]. These conditions can be mimicked *in vitro* by chemical inhibitors of the caspases or the IAPs, and as we will discuss below, also occur during certain infections.

Pathogen defense

While genetic studies have proven invaluable to our understanding of the signaling mechanisms of necroptosis, the question remains: why has necroptosis been selected for during evolution? RIPK3-deficient mice lack an overt phenotype, but are sensitive to many viral infections[14-17]. Furthermore, several pathogens have evolved mechanisms to antagonize the necroptotic pathway, indicating that disrupting RIPK3 activation is critical to successful infection by these organisms[15,18]. These findings are consistent with the idea that the core function of the necroptotic pathway lies in the host response to infection. Here we will discuss how pathogens trigger RIPK3-dependent responses, how they have evolved to evade those responses, and how type I IFN signaling, a well characterized innate immune program, plays a critical role in necroptosis.

Through the trap door: caspase inhibition and necroptosis induction in physiological settings

In vitro studies on mechanisms of RIPK3 activation usually require the use of caspase inhibitors, hinting at its physiological role. Caspase-dependent cell death is the most ancient form of programmed cell death, and evolutionary evidence implies that the intrinsic apoptotic pathway, which is responsible for developmental and homeostatic cell death, originated as a pathogen-sensing pathway[19]. Thus, caspase-dependent death was presumably the only form of programmed death that pathogens had to evade at one time. It is therefore not surprising that many pathogens encode caspase inhibitors

that allow for escape from apoptosis or the related caspase-dependent process of pyroptosis. However, as the RIP kinases evolved, it may be that a cell death function was gained by this pathway to induce programmed cell death when caspases were no longer active, giving the cell a trap door for pathogen clearance[20]. Vaccinia virus (VACV) was one of the first viruses shown to induce RIP3 activation[17]. VACV encodes a caspase inhibitor (B13R/Spi2) that sensitizes cells to programmed necrosis[21]. Induction of RIPK3-dependent effects are critical to host survival, as RIPK3-deficient mice succumb to VACV infection while WT mice are able to clear the infection. Presumably this is due to RIPK3's pro-death function, however other roles of RIPK3 (discussed later) have not been examined in this model. Infection of MLKL knockout animals with VACV would be informative to determine if necroptosis is the primary mediator of host survival.

Like viruses, many bacteria encode proteins that suppress caspase activation; for example, pathogenic *E. coli* produce NleB1, a type 3 secretion system (T3SS) effector that inhibits caspase-8 activity, blocking apoptosis in infected cells[22]. Furthermore, a component of bacterial cell wall, LPS, has been show to activate RIPK3 *in vitro* through a common TLR adaptor and RHIM-domain containing protein, Trif[23]. This combination of caspase inhibition and TLR stimulation would seem an ideal activator of necroptosis. However, RIPK3's role in bacterial infections is complicated, and a protective role for necroptosis during bacterial infection has not been clearly demonstrated. Given the existence of caspase inhibitors in bacteria, it may be that these pathogens also encode inhibitors of RHIM-dependent signaling. Analogous to effects observed in DNA viruses (discussed below), these inhibitors would effectively

defuse this pathway, eliminating differences observed in RIPK3-deficient settings unless the inhibitors were removed. Furthermore, unlike viruses, not all bacteria need to replicate inside a cell, making cell death a less valuable option for host defense. Indeed, there are examples of bacteria inducing cell death in immune cells to successfully evade the host response[24]. Finally, control of pathogenic bacteria seems to rely more readily on the lytic process of pyroptosis than on necroptosis[30].

While caspase-8 suppression is clearly an important sensitizer to necroptosis, it is likely not the only cellular context where RIPK3 can be active. When added to cells *in vitro,* TNF acts as a pro-survival signal unless caspase-8 is absent or inhibited. But injection of high doses of TNF into a mouse causes a sterile shock response, and surprisingly this is RIPK3-dependent[25] and partially MLKL-dependent[32]. How does TNF lead to a RIPK3-dependent response *in vivo* without caspase-suppression? There are a few hypotheses that could explain these observations. First, in a whole animal, there could be a subset of “necroptosis ready” cells in which caspase-8 activity is low, effectively priming them for RIPK3-activation. But perhaps a more intriguing hypothesis is the idea that mammals have evolved additional “trap doors” to trigger RIPK3 activation. An important component of caspase-8’s ability to suppress necroptosis is the upregulation of its binding partner, cFLIP[6]. cFLIP is a target of NF-kB signaling, and blocking transcription or translation of this protein can allow both necroptosis and caspase-8-driven apoptosis to occur. In this scenario, a failure of some cells to adequately upregulate cFLIP could explain their apparent susceptibility to RIPK3-dependent cell death during models of TNF septic shock, which could also induce apoptosis in some tissues. Perhaps in agreement with this idea, mice lacking both

caspase-8 and RIPK3 or MLKL display a significantly greater resistance to systemic TNF administration than mice lacking RIPK3 or MLKL alone[26].

The idea that failure to upregulate cFLIP or other survival factors could act as a key sensitizer to necroptosis may also apply to settings of pathogen infection. Many pathogens modify transcription and/or translation during their takeover of the cellular environment, and perturbations of cFLIP expression, or protein synthesis in general, may represent an additional "trap door" pathogens can fall into, triggering a RIPK3-dependent response. A possible (though unproven) example of this idea comes from work on influenza A virus (IAV). Infection with influenza has recently been shown to trigger necroptosis without the addition of a caspase inhibitor both *in vitro* and *in vivo*[16]*.* Interestingly, unlike other RIPK3-activating viruses, IAV has not been shown to encode its own endogenous caspase inhibitor to block caspase-8 activity and sensitize cells to a RIPK3-dependent response. Indeed, caspase-8 activity seems to be intact in IAV-infected cells, as deletion or knockdown of MLKL results in back-signaling from the RIP kinases and caspase-8-dependent apoptosis. What allows for RIPK3 activation in the presence of active caspase-8? IAV is known to block translation of cellular mRNAs[33], and perhaps this change in protein synthesis or changes in particular protein levels (like cFLIP) allows for RIPK3 activation. While caspase-8 suppression is the most well characterized "trapdoor" for pathogens, it seems probable that there are other cellular perturbations that lead to RIPK3 activation and necroptosis. What these cellular changes are and how they lead to RIPK3 activation remains to be explored.

RHIM-based viral evasion

Perhaps the most compelling evidence that the RIP kinase pathway plays an important role in viral defense is that viruses have evolved to counteract programmed necrosis. This has been most extensively characterized in DNA viruses. MCMV contains both a viral inhibitor of caspase activation (vICA) and a viral inhibitor of RHIM activation (vIRA)[15]. vICA blocks apoptosis, but is a double-edged sword for the virus, as it also blocks caspase-8 activity and sensitizes to necroptosis. However, vIRA contains its own RHIM domain which interferes with the interaction of RIPK3 with DAI, a RHIM-containing protein shown to be important in controlling herpesvirus infection through induction of necroptosis[14]. It seems likely that a proto-virus may have first acquired the ability to block apoptosis, which at one time might have been the primary cell death response to infection. However, as RIP kinases evolved and necroptosis emerged as an outcome of caspase suppression, the virus in turn had to counteract both apoptosis and necroptosis for its continued propagation. Intriguingly, HCMV seems to have evolved a different strategy to counteract necroptosis, as it does not encode a RHIM domain-containing protein like vIRA, but can still block necroptosis downstream of RIPK3 via an unknown mechanism[27]. This highlights not only the co-evolution of these DNA viruses with their hosts, but also the multitude of strategies a virus can take to block RIP3-dependent death (Figure 1). Like MCMV, herpes simplex viruses-1 and -2 (HSV-1/2) also encode RHIM-domain containing proteins (ICP6 and ICP10, respectively) that block necroptosis in human cells[28]. Interestingly, these proteins were previously shown to block caspase-8 activity, and so simultaneously sensitize cells to necroptosis, while blocking RIP3 activation. Again, we might hypothesize that HSV first acquired the ability to block caspase-8 dependent apoptosis, and then had to acquire the ability to block the

cell death trapdoor of necroptosis. But unlike MCMV, HSV uses one protein to block both cell death programs, likely constraining the evolution of this protein to retain both functions.

Surprisingly, ICP6 and ICP10 do not block necroptosis in murine cells, but rather activate RIPK3, highlighting a lack of species crossover for RHIM activity by this virus. The biochemistry and mechanism of RHIM-RHIM interactions and oligomerization are incompletely understood. Presently, we understand that RHIM interactions are critical for both negative regulation of RIPK3 activation, as well as oligomerization upon its activation[5,29]. Current models postulate that conformational changes following post-translational modification or protein binding lead to RHIM exposure and initiate the formation of RHIM-containing complexes. But it remains unclear whether all RHIMs are able to participate in such complexes, and whether certain RHIMs are intrinsically stiochiometrically favored within the RHIM amyloid. While it is clear that RIPK1's RHIM allows for bridging between RIPK3 and caspase-8, it is unclear how other RHIM-containing proteins, including RHIM-containing viral inhibitors, restrict RIPK3 activation. Do these proteins target RIPK3 for degradation or sterically hinder the formation of protein complexes? As the core interaction-determining region of the RHIM is very small, perhaps it simply initiates protein associations while the rest of the protein dictates whether oligomerization is propagated or blocked. The result of this interaction is species-specific, as HSV can only block RIPK3 activation in human cells, its natural host. This raises the possibility that only the correct interaction will block oligomerization, while other interactions through a RHIM might propagate the oligomer.

Further investigations into the biochemistry of RHIM interactions might be enlightening on this front.

Necroptosis as an arm of the interferon-driven antiviral response

Type I interferons are a set of secreted cytokines capable of upregulating hundreds of genes that induce an antiviral state within cells. Interestingly, macrophages deficient in type I IFN signaling could not undergo necroptosis following treatment with RIPK3 activators, such as TNF and toll-like receptor ligands[30]. This finding is surprising, as the mechanisms of RIPK3 activation for these ligands are known, and relatively linear, with no known role for interferons. Though MLKL is IFN-inducible, protein levels are similar between WT and IFNAR-deficient cells, pointing to additional IFN-dependent control of necroptosis that has yet to be uncovered. Perhaps some additional interferon-stimulated gene (ISG) plays a critical, yet undefined general role in necroptosis. Understanding why type I IFN contributes to induction of necroptosis downstream of all known RIPK3-activating ligands remains an intriguing open question in the field.

Additionally, IFN itself seems to be capable of inducing necroptosis in cells lacking caspase-8 function[31], though the mechanism of how this occurs remains murky. While PKR, an RNA sensor, has been implicated in this process, subsequent work found no role for PKR in IFN-induced necroptosis[30]. An intriguing alternative hypothesis on how type I IFN might cause necroptosis is by the upregulation of DAI, a highly IFN-responsive gene that is one of four RHIM-domain containing proteins in mammals[32]. DAI is known to be an important sensor of herpesvirus infections, and can directly interact with RIPK3, leading to its activation and necroptosis[14]. While DAI has been

described as a DNA sensor[40], it has never been shown to bind DNA during a herpesvirus infection, nor to trigger necroptosis following introduction of a pure DNA ligand to the cytosol. Furthermore, DAI has most recently been shown to play an important role in triggering RIPK3 activation and cell death during infection with an RNA virus, influenza virus[41]. What does DAI sense – DNA, RNA, or something else? Perhaps expression of DAI over a certain threshold can lead to spontaneous interaction with RIPK3, or perhaps DAI can be activated by an endogenous ligand, either a nucleic acid motif or a protein. If this were true, DAI could be responsible for IFN-induced necroptosis, either due to exogenous addition or during the course of viral infections. A better understanding of how IFN signaling interfaces with and sensitizes to necroptosis will likely be forthcoming, and may support the idea that necroptotic cell death is a component of the IFN-driven antiviral response.

Evolutionary perspectives: inflammation before death

Much of the research surrounding RIPK3-dependent signaling has revolved around its pro-death role. But emerging evidence shows that RIPK3 may have non-cell death functions in pro-inflammatory signaling. Perhaps we should not be so surprised that RIPK3 has other functions; it is strongly tied to a pathway that has its roots in inflammatory signaling. Cell death may therefore represent a more recently acquired function of the RIP kinase pathway.

A homologue of RIPK3 is present in lampreys[33], indicating that it emerged, likely as a gene duplication event, early in the vertebrate lineage. But much of the pathway involved in RIP kinase signaling has even more ancient roots. Drosophila have

homologs of many members of the TNF-driven proinflammatory pathway, including a RHIM domain-containing RIPK1-like molecule, imd. Remarkably similar to mammalian RIPK1, imd associates with the Drosophila FADD homolog and a caspase dredd, and activates Relish, the fly equivalent of NF-kB, through coordinated signaling of IKK homologs and ubiquitin43. Interestingly, the imd pathway senses bacterial peptitoglycans and promotes the release of antimicrobial molecules, suggesting that this pathway has an ancient role in host defense. Glycan sensing in mammals is accomplished in part by the NOD receptors which converge on activation of another RIPK family member, RIPK2[34]. This suggests additional divergent evolution in these pathways between fly and mammals; NOD/RIP2 maintained the glycan sensing ability of the imd pathway, while the ancestral signaling components were re-configured into the RIP1/RIP3 pathway which now responds to other signals. Similar to the mammalian TNF response, under certain stimuli, the imd pathway can lead to caspase-dependent cell death45, mirroring caspase-8-dependent apoptosis. But Drosophila do not have a RIPK3 homolog, nor do they seem to have a caspase-independent cell death program.

So why do mammals have RIPK3? Perhaps one clue lies in its sequence. Comparison of gene sequences between evolutionarily related species allows measurement of the rate with which a given gene is undergoing non-synonymous mutation, which in turn allows estimation of the evolutionary pressure being applied to a given protein. Most often, this pressure represents efforts by pathogens to antagonize a given protein, conferring evolutionary advantage on mutations that allow escape from that antagonism. Applying this measure of “positive selection” to the RIPKs reveals that while RIPK3 is rapidly evolving under positive selection in primates, RIPK1 is not (M.

Patel and H. Malik, personal communication). Gene duplication events can allow proteins more flexibility in their evolution while maintaining their core function. In mammals, loss of RIPK1 is perinatally lethal due to unrestrained programmed cell death[4,35], and this likely constrains the ability of RIPK1 to evolve in the face of invading pathogens. Perhaps the emergence of RIPK3 allowed the pathway to rapidly evolve its additional pro-death function without losing the indispensable functions of RIPK1 in the process. Certainly during evolutionary time, animals were being exposed to various pathogens, requiring nimble evolution of innate immune responses. Duplication and subsequent neofunctionalization of the RIP kinases may have helped our animal ancestors survive these infections.

One of the roles RIPK3 has evolved is the ability to induce necroptosis. But if its roots are within this proinflammatory signaling platform, it may have other non-cell death roles. Indeed, many death-independent roles of RIPK3 are beginning to be described. Bone marrow derived macrophages stimulated with LPS and zVAD produce TNFa in a RIPK1 and RIPK3-dependent manner[36]. However, this effect does not require the expression of MLKL, indicating that these effects are unrelated to RIPK3's ability to induce cell death. While the biological circumstances in which LPS-driven, RIPK3-dependent inflammatory transcription might be important remain unclear, these findings clearly indicate a death-independent function for RIPK3. Furthermore, RIPK3-deficient mice show more protection from disease models of kidney ischemia-reperfusion and A20-deficiency than MLKL KO animals[26], implying functions of RIPK3 in these models that extend beyond MLKL activation and necroptosis. Intriguingly, direct activation of RIPK3 has been shown to produce immunostimulatory cytokines that allow for better

cross priming of T cells[37], indicating that one of RIPK3's non-death roles may be linking the innate and adaptive immune responses. Interestingly, production of these cytokines requires the RHIM domain of RIPK3 in order to engage RIPK1 and NF-kB signaling, somewhat backwards from the canonical RIPK1 to RIPK3 signal. However, as RIPK3 can be activated independently of RIPK1 by other RHIM-containing proteins[14,32], this "backwards" interaction could allow RIPK3 to engage the proinflammatory capacity of RIPK1 outside of traditional receptor-driven signaling, which may play an important inflammatory role during infection. Additionally, RIPK3 activation during viral infection has been shown to promote the activation of the NLRP3 inflammasome and processing of IL-1β[38], however it's unclear whether this is a cell death-independent role of RIPK3. Regardless, perhaps one early role of RIPK3 was linking antiviral signaling with proinflammatory cytokine production. While these results are illuminating, understanding the capacity and mechanism of RIPK3's coordination of both cytokine production and cell death is still in its infancy. As many disease models have implicated RIPK3 involvement, either in clearance of infection or propagation of inflammatory effects, it will be important to further clarify whether the pathology of these models is driven by a pro-death or non-death role of the RIP kinases.

Concluding remarks

Evolutionary studies imply that while the ancestral RIP kinase pathway did not contain a functional RIPK3-like molecule or induce caspase-independent cell death, evolution favored these adaptations. For what purpose were they added? Examining what we know about RIPK3-dependent signaling is suggestive of an answer.

Necroptosis is triggered upon infection by several viral pathogens *in vitro*, and mice lacking RIPK3 succumb to many of these pathogens more readily than wild-type animals. Furthermore, it appears that IFN signaling—a master regulator of the anti-viral response—is required for necroptosis in at least some cells, though the mechanism by which IFN and necroptosis are connected is not clear. These findings support the idea discussed in the introduction that RIPK activation during viral infection triggers inflammatory cell death, eliminating infected cells and promoting inflammation and immunity. Notably however, clear demonstration of necroptosis *in vivo* during infection, and precisely how this cell death program might promote host defense, remains elusive.

While much has been learned since the discovery of RIPK3, many questions remain about its activation. Are there other sensitizers to RIPK3 activation besides caspase-8 inhibition? It seems likely that some kind of transcription or translation perturbation could also lead to activation, but the nature of these interactions is not well understood, particularly in an *in vivo* setting. How exactly does IFN induce RIPK3 activation? Furthermore, do ligands capable of activating other antiviral IFN-production pathways, such as the cGAS-STING or RIG-I-like receptor pathways, also activate RIPK3? If IFN can induce necroptosis, it should follow that inducers of its expression might also lead to a similar response, expanding the possibility of RIPK3-dependent responses to other infections and disease models. It is possible that these ligands could work through a universal mechanism of RIPK3 activation that could shed light on why IFN signaling seems to be broadly required for necroptosis, at least in some cell types.

While RIPK3-dependent cell death has been the primary focus of research into its function, we increasingly appreciate that this is not the only function of RIPK3. It is

becoming apparent that the RIP kinase pathway coordinates both the production of proinflammatory cytokines and cell death. Studying the non-death functions of RIPK3 remains difficult, due to the nature of the pathway it evolved from; RIPK1 has its own transcription effects, many of which are independent from RIPK3. Further, recent work indicates that caspase-8 can participate in cytokine production as well[39]. And yet still the question remains, how and why exactly did RIPK3 evolve to be activated when caspase-8 is suppressed? Perhaps this is simply an additional checkpoint to ensure that necroptosis does not happen when apoptosis could be favored, as necroptosis might lead to unnecessary inflammation and immune response. However, there may be other possibilities related to the non-death roles of these proteins. Perhaps RIPK3 activation leads to a distinctly different set of cytokines that instruct an inflammatory response in ways that caspase-8 or RIPK1-dependent cytokines do not. Comparison of RIPK3-deficient and MLKL-deficient mice seems to be a viable way to determine which responses are dependent on cell death, rather than the inflammatory effects of RIPK3. Untangling RIPK3's role in inflammation from the inflammatory capacity of caspase-8 and RIPK1 remains a challenging task, but one that will illuminate the evolutionary emergence and function of this novel cell death pathway.

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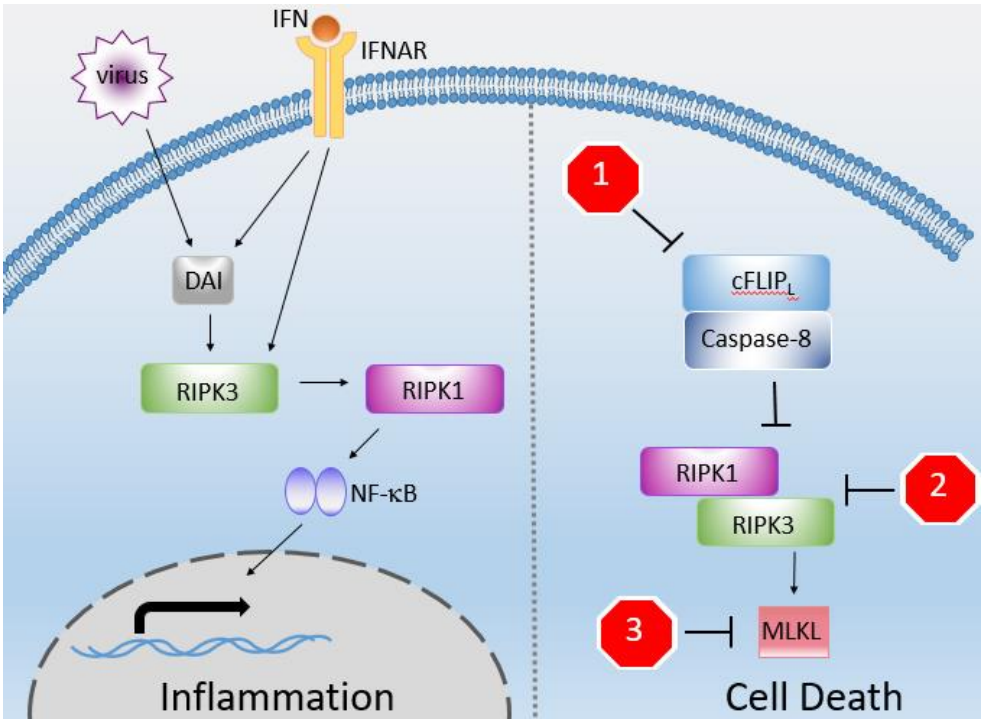

Stop sign	Interfering proteins (pathogen)
1	ICP6 (HSV-1)[35], ICP10 (HSV-2)[35], vICA (CMV)[16], vFLIP (KSHV, MCV), CrmA (Cowpox), B13R (VACV)[25], E3 (Adenovirus)[23], Serp2 (Myxoma virus)[23], E6 (HPV-16)[23]
2	ICP6 (HSV-1)[35], ICP10 (HSV-2)[35], vIRA/M45 (mCMV)[16]
3	IE1 (hCMV)[34]

Figure 1: RIPK3 has both death and non-death roles

RIPK3 can mediate both inflammation and cell death. While the cytokine-producing functions of RIPK1 and caspase-8 are better understood, RIPK3's roles are still under investigation. Viruses, potentially through DAI or IFN, can directly activate RIPK3, allowing RIPK3-dependent cytokine production, likely through interactions with RIPK1 or

caspase-8. Inflammation may be the more ancient function of the RIP kinase pathway, and begs the question: can this occur independently of RIPK3's cell death role? RIPK3's cell death role is more established, and many pathogens engage with diverse proteins associated with necroptosis. Some pathogens sensitize to necroptosis by blocking the caspase-8/FLIP heterodimer (stop sign 1). Others block necrosome formation (stop sign 2). And finally, others block downstream of RIPK3 activation in a less-well understood fashion, potentially involving blockade of MLKL oligomerization, access to the cell membrane, or perturbation of yet-to-be-described cofactors (stop sign 3). These pathogen interactions have allowed greater understanding of how the pathway works and its physiological role in pathogen defense.

Chapter 3: Intracellular nucleic acid sensing triggers necroptosis through synergistic type-I interferon and TNF signaling

Introduction:

This chapter contains my manuscript for publication based of the bulk of my research. This research was undertaken to answer if and how intracellular DNA and RNA could induce necroptosis, and whether IFN signaling played a role in any cell death seen, as IFN was discovered to be a major player in the activation of necroptosis shortly after this research was started. Additionally, it explores the mechanisms of nucleic acid-induced necroptosis *in vivo* using a STING agonist in a murine model. The findings advance our understandings of the connections between nucleic acid sensing and cell death, and have implications for the future development of STING agonists in the clinic.

Manuscript:

Intracellular nucleic acid sensing triggers necroptosis through synergistic type-I interferon and TNF signaling

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Summary: The sensing of cytosolic nucleic acids, a signature of viral infection, can trigger necroptotic cell death through the synergistic action of the cytokines type-I interferon and TNF.

Abstract

The sensing of viral nucleic acids within the cytosol is essential for the induction of an innate immune response following virus infection. However, this sensing often occurs within cells that have already been infected. The death of these infected cells can be beneficial to the host by eliminating the virus's replicative niche and facilitating the release of inflammatory mediators, and several connections between innate sensing of viral infection and programmed cell death have been reported. Here, we show that sensing of intracellular DNA or RNA by cGAS-STING or RIG-I-MAVS, respectively, can lead to the activation of RIPK3 and necroptosis. Notably, this requires signaling through both type I interferon (IFN) and tumor necrosis factor (TNF) receptors, revealing a unique synergy between these signaling pathways to induce cell death. Furthermore, we show that hyper-activation of STING *in vivo* leads to a shock-like phenotype, the mortality from which requires activation of the necroptotic pathway and synergistic IFN

and TNF signaling, demonstrating that necroptosis is an important outcome of STING signaling *in vivo*. During infection, necroptosis represents an additional arm of the antiviral response.

Introduction

The induction of programmed cell death is an important process for maintaining tissue homeostasis, as well as improving immune system function. While billions of cells in our bodies die each day by apoptosis, the induction of lytic cell death programs during infection more readily instruct and aid our immune system. One such cell death program is programmed necrosis, or necroptosis, which is both morphologically and mechanistically distinct from apoptosis, and requires the activation of receptor interacting protein kinases 1 and 3 (RIPK1 and RIPK3)(*1*). Notably, RIPK3-deficient animals display increased susceptibility to a variety of viral infections *(2-6)* highlighting the role of this pathway in antiviral host defense. Furthermore, cells deficient in type-I interferon (hereafter IFN) signaling, an essential component of antiviral defense, fail to induce necroptosis following treatment with a variety of RIPK3-activating stimuli(*7*), suggesting that necroptosis may constitute an arm of the IFN-driven antiviral response.

The sensing of foreign nucleic acids is a key initiating event in the innate immune response to viral infection. The presence of DNA in the cytosol is one such signature of infection. In most cell types, cytosolic DNA activates the cGAS-STING pathway(*8*). Briefly, cGAS senses DNA and synthesizes the secondary messenger cyclic GMP-AMP (cGAMP)(*9*), which binds directly to STING(*10*), leading to activation of TBK-1 and IRF3-dependent transcription of IFNβ(*11*). The production and signaling of type I IFN

leads to the induction of hundreds of IFN-stimulated genes (ISGs), inducing an antiviral state. As such, cGAS or STING-deficient mice are highly susceptible infection by various DNA viruses (*12-14*). Intriguingly, many of these viruses also lead to significant mortality in RIPK3-deficient animals(*2*, *3*, *5*, *15*). A recent study found that murine gammaherpesvirus-68 (MHV68) could induce necroptosis in L929 cells, and that this response was prevented by knockdown of STING (*16*). However, mechanistic and physiological links between STING signaling and necroptosis remain unclear.

Here we show that the sensing of cytosolic DNA induces programmed necrosis through the activation of the cGAS-STING pathway. Interestingly, robust activation of cell death by this pathway requires not only type I IFN signaling, but also an intact TNF signaling pathway. Furthermore, we show that RNA ligands acting through the sensors TLR3 or RIG-I also require both IFN and TNF signaling to induce necroptosis, highlighting the essential synergy between these signaling pathways in response to a variety of innate immune stimuli. Finally, we find that *in vivo* administration of a STING agonist leads to a fatal, shock-like inflammatory disease in mice. Mortality associated with this disease requires both TNF and IFN signaling, and is significantly rescued in animals lacking components of the necroptotic pathway. These results indicate that multiple nucleotide sensing pathways can trigger necroptosis through the synergistic action of the IFN and TNF, and that this signaling can mediate lethal shock in mice.

Results

Introduction of DNA into the cytosol can trigger necroptosis

We sought to understand the cellular response to cytosolic DNA, using primary bone marrow-derived macrophages (BMDM) as an experimental system. We observed that introduction of DNA into the cytosol led to rapid and robust cell death in primary murine BMDM. As has been previously described(*17*), this cell death response was AIM2- and caspase-1-dependent and morphologically consistent with inflammasome-induced pyroptosis (Fig. 1, A and B). Unexpectedly however, upon addition of the pan-caspase inhibitor zVAD-FMK, macrophages underwent slower but equally robust cell death in response to DNA transfection (Fig. 1C). This cell death was morphologically consistent with programmed necrosis (Fig. 1B), a lytic cell death program dependent on activation of RIPK3. Indeed, we observed that RIPK3-deficient macrophages were resistant to cell death in response to DNA transfection in combination with zVAD-FMK (Fig. 1C). Furthermore, transfection with DNA lead to phosphorylation of RIPK3 (Fig. 1D), confirming that DNA stimulation can lead to necroptosis when caspases are suppressed.

Given the robust AIM2 dependent response observed upon introduction of cytosolic DNA alone, we next sought to determine whether components of the AIM2 inflammasome contributed to the cell death we observed upon DNA transfection combined with caspase inhibition. To assess this, we transfected DNA into macrophages lacking the inflammasome component AIM2, and measured their cell death response over time. While pyroptosis could not occur in macrophages missing key inflammasome components, these cells could still undergo necroptosis in response to cytosolic DNA (Fig. 1E) in combination with caspase inhibition. These findings

indicate that when AIM2-dependent pyroptosis is prevented, cytosolic DNA can trigger necroptosis via a distinct signaling pathway.

Cytosolic DNA triggers necroptosis via the cGAS-STING pathway

In an effort to identify the pathway by which cytosolic DNA causes necroptosis, we tested macrophages lacking candidate DNA sensors. A proposed DNA sensor, DAI (encoded by *Zbp1*), has been shown to be important for the induction of necroptosis during herpesvirus infections(*2*). However, DAI-deficient macrophages were still able to undergo necroptosis following DNA transfection (Fig. 2A), implying that DAI is not required for the induction of necroptosis by a pure DNA ligand. This finding is consistent with recent reports that DAI may sense RNA ligands*(18, 19)*.

The DNA sensor cGAS (encoded by *Mb21d1*) and its signaling adaptor STING (encoded by *Tmem173*) are required for the induction of type-I IFN following detection of DNA in the cytosol. To determine whether activation of the cGAS-STING pathway could also lead to the induction of necroptosis, we transfected DNA into macrophages deficient for either protein. When caspases were suppressed, we found that macrophages lacking cGAS or STING failed to undergo necroptosis after cytosolic DNA stimulation (Fig. 2B), confirming that this pathway is required for the necroptotic response to DNA ligands. cGAS produces a secondary messenger, cyclic GMP-AMP or cGAMP, which binds to and activates STING. We sought to understand whether, like cytosolic DNA, cGAMP could also lead to the induction of necroptosis. Indeed, we observed that upon transfection of cGAMP into macrophages, these cells underwent RIPK3-dependent cell death when caspases were suppressed (Fig. 2C). We observed

that like DNA, cGAMP-induced necroptosis required STING (Fig. 2D). However, unlike DNA, cGAMP treatment in the absence of caspase inhibition did not trigger pyroptosis (Fig. 2, C and D). Together, these data indicate that activation of the cGAS-STING pathway can lead to necroptosis.

STING-dependent IFN production is necessary but not sufficient to induce necroptosis

The canonical function of the cGAS-STING pathway is the production of type I IFN, and IFN treatment alone has been shown to induce necroptosis under certain conditions*(20)*. We therefore tested whether autocrine or paracrine signaling by IFN produced upon stimulation of the cGAS-STING pathway was necessary for the induction of necroptosis. Following transfection with CT DNA or cGAMP, we observed that IFNαR1-deficient macrophages, which do not respond to IFN, were resistant to necroptosis (Fig. 3, A and B). These results indicate IFN signaling is required to induce necroptosis following cGAS-STING stimulation, echoing results for other RIPK3-activating ligands like TNF, LPS, and poly(I:C)(*7*). Consistently with this finding, we observed that both STAT1 and STAT2, important signaling molecules downstream of IFN receptor stimulation, were required for cGAMP-induced necroptosis (Fig. S1A). We next tested whether IFN signaling was sufficient to induce necroptosis in macrophages. We found that treatment of WT, but not *Ripk3*$^{-/-}$ primary macrophages with recombinant IFN in combination with zVAD-FMK led to modest necroptotic cell death, and that unlike the robust and consistent necroptotic response observed upon STING activation, the magnitude of cell death triggered by recombinant IFN was highly variable between experiments. (Fig. 3C and fig. S1B). Notably however, the necroptotic response to even

supraphysiological doses of recombinant IFN did not recapitulate that observed upon STING activation. Together, these data indicate that IFN signaling is necessary but not sufficient for the necroptotic response we observe upon activation of the cGAS-STING pathway.

STING-dependent TNF production is required for induction of necroptosis

Since IFN alone was unable to fully recapitulate the necroptotic response observed upon treatment with DNA or cGAMP, we hypothesized that other STING-dependent signaling might contribute to the activation of the necroptotic cell death program. To identify such a signal, we transfected WT macrophages as well as macrophages lacking STING with cGAMP and assessed cytokine production from these cells by Luminex. We observed that in WT macrophages, but not in *Tmem173*$^{-/-}$ cells, cGAMP treatment led to upregulation of TNF, a cytokine well characterized for its ability to induce programmed necrosis (Fig. 4A). This finding indicated that, as has been shown in other systems*(21, 22)*, STING activation can lead to production of TNF as well as type-I IFN.

We next sought to determine whether STING-dependent production of TNF was required for the cell death response to DNA. To test this, we transfected WT macrophages, as well as cells deficient for TNF, TNFR1 (*Tnfrsf1a)*, or TNFR2 (*Tnfrsf1b)*, with cGAMP. When caspases were suppressed, macrophages deficient in TNF or either of its receptors failed to undergo necroptosis after cGAMP stimulation (Fig. 4B and fig. S2A), demonstrating that TNF is essential for robust and efficient necroptosis following activation of the cGAS-STING pathway. While TNFR1 is well

characterized as a driver of RIPK1 activation*(23)*, we sought to understand the observed requirement for TNFR2 in this setting. One known effect of TNFR2 signaling is the degradation of the cellular inhibitors of apoptosis (cIAPs), which restrict necrosome formation*(24)*, and this de-repression has been reported to be required in combination with RIPK1 activation by TNFR1 to allow necroptosis to proceed*(25)*. To assess this, TNFR1 and TNFR2-deficient cells were treated with zVAD and the IAP antagonist BV6. We observed that cells deficient in TNFR2, but not TNFR1, were sensitive to necroptosis in these conditions (Fig. S2B), consistent with the idea that TNFR2 signaling in this setting acts to de-repress necroptosis by degrading the cIAPs.

As some components of the necroptotic pathway are upregulated in response to IFN or TNF signaling, we sought to ensure that failure to induce necroptosis in TNF or IFNαR1-deficient macrophages was not due to cell intrinsic defect in tonic protein levels. To determine if necroptotic pathway components were present at comparable levels in these cells, WT, *Tnf*$^{-/-}$, and *Ifnar1*$^{-/-}$ macrophages were blotted for RIPK1, Caspase-8, RIPK3, and MLKL. Levels of these proteins were comparable between WT, *Tnf*$^{-/-}$ and *Ifnar1*$^{-/-}$ cells (Fig 4C), indicating that the resistance of cells lacking TNF or IFN signaling does not arise from a failure to express components of the necroptotic pathway. Given the profound resistance to STING-induced necroptosis observed in both *Tnf*$^{-/-}$ and *Ifnar1*$^{-/-}$ cells, we sought to test whether lack of one of these signaling pathways affected the other. However, we found that upregulation of a panel of IFN-responsive genes by LPS, cGAMP, or recombinant IFN was intact in *Tnf*$^{-/-}$ macrophages (Fig. 4D), indicating that the IFN pathway is intact in these cells. Furthermore, while we saw that cGAMP treatment led to upregulation of TNF message, recombinant IFN treatment failed to

upregulate TNF in WT macrophages (Fig. 4E), indicating that IFN signaling alone is not sufficient to initiate TNF signaling. Together, these data indicate that TNF signaling in response to activation of the cGAS-STING pathway is required for necroptosis, and that TNF signaling acts in concert with, but independently from, the IFN pathway to trigger necroptosis in this setting.

Synergistic IFN and TNF signaling is also required for RIG-I and TLR3-induced necroptosis

Co-activation of both IFN and TNF production is a motif observed downstream of multiple innate immune sensors. Because we observed synergistic signaling of IFN and TNF to trigger necroptosis upon activation of the cGAS-STING pathway, we tested whether similar synergy occurred downstream of other nucleotide sensing pathways. To do this, we assessed the response to 5'-triphosphate RNA, a ligand for the cytosolic RNA sensor RIG-I, and to poly(I:C), a ligand for toll-like receptor 3 (TLR3). Upon transfection of primary macrophages with 5'-triphosphate RNA or poly(I:C) in combination with caspase suppression, we observed robust RIPK3-dependent cell death (Fig. 5A). As expected, necroptosis induced by RIG-I ligand required MAVS, the downstream signaling partner of RIG-I (Fig. S3A), confirming that this stimulus acted through the RIG-I-MAVS pathway. Analogously, and in accordance with previous studies*(7, 26)*, we observed that poly(I:C) stimulation induced necroptosis in WT but not TLR3- or IFNαR1-deficient macrophages (Fig. S3, B and C). When caspases were inhibited, RIG-I ligand similarly required IFNαR1 to induce necroptosis (Fig. 5B),

indicating that, like TLR signaling, RIG-I induced necroptosis required an intact IFN pathway.

In order to determine whether these RNA ligands required TNF for efficient necroptosis, we stimulated WT and *Tnf*$^{-/-}$ macrophages with either 5' triphosphate or poly(I:C). We found that TNF-deficient macrophages failed to undergo necroptosis following either poly(I:C) or RIG-I ligand stimulation (Fig. 5C), confirming that like DNA, RNA ligands require intact TNF signaling to induce necroptosis. Consistent with this, we saw that phosphorylation of RIPK3 was absent following poly(I:C) treatment in *Ifnar*$^{-/-}$ macrophages, and significantly reduced in *Tnf*$^{-/-}$ cells (Fig. 5D), indicating a requirement for both TNF and IFN signaling downstream of poly(I:C) stimulation.

Finally, we sought to determine whether the moderate levels of cell death seen in WT macrophages following high dose IFN treatment were also dependent on the presence of TNF. To test this, we treated WT and TNF-deficient macrophages with a high dose of IFN and inhibited caspases. We found that *Tnf*$^{-/-}$ macrophages were completely resistant to IFN-induced necroptosis (Fig. 5E), indicating that even necroptosis induced by direct IFN stimulation requires TNF production and signaling. Taken together, these data indicate a broad requirement for synergy between IFN and TNF signaling for the induction of necroptosis downstream of multiple innate immune sensing pathways.

STING agonists induce sterile shock in mice

It has previously been established that activation of RIPK1 and RIPK3 contributes to the mortality associated with sterile shock models following administration

of high doses of TNF*(27)*. Because we observed a requirement for STING-dependent TNF production for the induction of necroptosis *in vitro*, we wondered whether systemic STING activation *in vivo* might also lead to RIPK3-dependent pathology. Notably, STING agonists are currently in clinical development as tumor immunotherapy agents, but potential adverse responses to systemic administration of these agents have not been studied. DMXAA is a potent activator of murine STING*(28)* that has been shown to have anti-tumor properties in mouse models*(29, 30)*. Consistent with its role as a STING agonist, we found that DMXAA, in combination with zVAD, induced robust necroptosis in primary macrophages *in vitro* in a STING and RIPK3-dependent manner (Fig. S4A), analogous to the effects of cGAMP and zVAD. To assess the systemic response to STING activation, we administered DMXAA intraperitoneally and monitored mice for clinical signs of shock, such as hypothermia, lethargy, ocular secretions, and the production of pro-inflammatory cytokines. We found that DMXAA induced significant mortality in C57BL/6J (B6/J WT) animals, while STING-deficient animals were completely resistant to mortality (Fig. 6A). Even more strikingly, STING-deficient mice were protected from all observable signs of shock, and serum collected from these mice lacked detectible levels of the pro-inflammatory cytokine interleukin-6 (IL-6) (Fig. 6A and fig. S4, B and C). These findings demonstrate that systemic DMXAA administration causes shock-like symptoms, and that this response is wholly dependent on STING signaling.

Systemic shock induced by STING activation engages TNF, IFN, and necroptotic signaling

We next sought to test whether the sterile shock and mortality triggered by systemic STING activation *in vivo* involved engagement of TNF and/or IFN signaling. To do this, we administered DMXAA to *Tnf*$^{-/-}$ and *Ifnar1*$^{-/-}$ animals. Whereas a majority of WT animals succumbed to disease within 24 hours, both *Tnf*$^{-/-}$ and *Ifnar1*$^{-/-}$ animals were completely resistant to mortality following DMXAA injection (Fig. 6B). Furthermore, mice lacking TNF or IFNAR1 showed a reduction in the inflammatory marker IL-6 upon systemic STING activation, though this reached statistical significance only in the case of TNF knockout (Fig. 6B and fig. S4C).

Given the synergy observed between IFN and TNF signaling *in vitro*, together with our finding that both pathways are involved in STING-driven shock *in vivo*, we next tested whether engagement of necroptotic signaling plays a role in the pathology observed upon DMXAA administration. To do this, we administered DMXAA to animals lacking either MLKL or RIPK3. *Mlkl*$^{-/-}$ animals were also significantly rescued from the lethal effects of STING activation, though not completely resistant (Fig. 5C). *Ripk3*$^{-/-}$ mice are backcrossed to the C57BL/6N strain background, while other mice used in this study are congenic with the C57BL/6J background. Because of reported differences between these sub-strains in other models of sterile shock*(31)*, we compared *Ripk3*$^{-/-}$ mice to littermate-matched WT or heterozygous animals. Both RIPK3-deficient and heterozygous animals were significantly rescued from mortality, while DMXAA was uniformly lethal to their wild-type littermates (Fig. 6D). Interestingly, whereas C57BL/6J mice have been shown to be more susceptible to recombinant TNF injection than C57BL/6N mice*(31)*, we found the opposite following DMXAA administration (Fig. 6A-D). Notably, we found that serum from mice lacking RIPK3 or MLKL had levels of the

pro-inflammatory cytokine IL-6 comparable to those observed in WT mice following DMXAA treatment (Fig. 6 C and D), suggesting that while the pathological response to inflammatory signaling is attenuated in these mice, the inflammatory signals themselves remain. Together, our findings indicate that activation of the cGAS-STING pathway *in vivo* causes activation of both IFN and TNF signaling, and that synergy between these pathways engages RIPK3 and MLKL to cause lethal shock.

Discussion

The sensing of foreign nucleic acids within the cell is a key initiator of the immune response to invading pathogens, and it can trigger diverse outcomes including both transcriptional changes and cell death. We show that following DNA detection, the cGAS-STING pathway can trigger necroptosis in primary macrophages when caspases are suppressed. Notably, this cell death response requires STING-dependent production of both type-I interferon and TNF, and the induction of necroptosis by STING activation involves reciprocal, synergistic signaling by these two pathways. Furthermore, as these signaling pathways are both activated downstream of other ligands, we show that RNA detection though both the RIG-I-MAVS pathway and the TLR3 pathway can trigger RIPK3 activation via a similar mechanism when caspases are suppressed, again requiring synergistic signaling through IFN and TNF.

The cGAS-STING pathway has been shown to play a critical role in the response to numerous DNA virus infections, through the production of type I IFN and resulting antiviral state(*8*). Viruses such as vaccinia trigger STING activation(*13*) but also encode caspase inhibitors that have been shown to inhibit caspase-8 and lead to RIPK3

activation*(32)*. While STING-dependent IFN production by infected cells may lead to the induction of an antiviral state in neighboring cells by paracrine signaling, our data would suggest that it may also act in an autocrine manner to induce death of the infected cell itself, thereby eliminating the replicative niche of the virus. This implies that RIPK3 activation represents an arm of the IFN-driven antiviral response, triggered only when the cell is exposed to coincident IFN and TNF signaling, as well as viral caspase suppression. This requirement may allow for communication of the need for an antiviral response through paracrine signaling, and concurrent elimination of the infected cells themselves.

In addition to its antiviral role, recent work has established STING as a mediator of beneficial anti-tumor immune responses*(33)*. DMXAA was a clinical failure, because it activates only murine, but not human, STING*(28)*. However, stabilized cyclic dinucleotide compounds that activate human STING are currently in clinical trials as tumor immunotherapy agents. These compounds require STING for their efficacy, and lead to the production of both TNF and IFN*(30)*. Our data indicate that dose-limiting toxicity of these agents likely involves engagement of the necroptotic pathway. While the long-term efficacy and toxicity of these compounds at clinically relevant doses has not yet been established, our findings indicate that modulation of the necroptotic pathway may help to alleviate any shock-like adverse reactions to these drugs. Conversely, it is unknown whether the antitumor effects of cyclic dinucleotides involve activation of RIPK3 downstream of STING signaling within the tumor microenvironment. It is possible that RIPK3 engagement and necroptosis contributes to the efficacy of STING agonists, as antigens derived from necroptotic cells activate T cell responses

more readily than those derived from apoptotic cells and might better inform a cellular immune response to tumors*(34)*.

A surprising facet of our data is the finding that both TNF and IFN signaling are required for induction of necroptosis by stimulation of TLR3, a pathway in which the RHIM-containing adapter TRIF is able to directly engage the RIP kinases*(26, 35)*. This requirement is not explained by TNF or IFN-mediated induction of known components of this signaling pathway. Furthermore, we show that necroptosis triggered by IFN signaling in the absence of pathogen-associated ligands requires TNF signaling, despite a lack of TNF induction upon IFN treatment. Thus, at least in this setting, it appears that tonic levels of TNF signaling are required to maintain cells in a necroptosis-competent state. This state likely reflects a combination of RIPK1 activation via TNFR1, and control of cIAP levels via TNFR2. How TNF and IFN interface in this setting remains to be understood. Future work will assess the role of these two pathways in the induction of necroptosis in infection and co-infection models where both IFN and TNF signaling is critical for pathogen clearance.

Materials and Methods

Cell culture, transfection protocol, and reagents

Macrophages were differentiated for seven days from whole bone marrow in RMPI (HyClone) with 10% CMG12-14 supernatant, 10% FBS (Gemini), 100 units/ml penicillin/streptomycin (HyClone), 2mM glutamine (HyClone), 50 mM beta-mercaptoethanol, and 10 mM HEPES (HyClone). Macrophages were lifted with 4mM EDTA in PBS, counted, and plated at 150,000 cells/well in 24 well plates. Cell death

assays were done using the IncuCyte bioimaging platform (Essen). Death was measured by the incorporation of Sytox green (Life Tech) and normalized to total cell count using Syto green (Life Tech). zVAD-FMK (SM Biologicals) was used at a concentration between 25-50 μM depending on application. Transfections were done at a 1:1 ratio with Lipofectamine 2000 (Life Tech) and 2 μg/ml of calf thymus DNA (Sigma) or cGAMP (Invivogen). Poly(I:C) (EMD Millipore) was added directly to the media at 1 μg/ml. RIG-I ligand (5' triphosphate RNA, made in house) was transfected at concentration of 1ug/ml. High dose IFN A (PBL Assay Science) for cell death assay was 100 units/ml, based on the specific activity of each lot. For Luminex panels, macrophages were transfected with each ligand and supernatants were collected after 6 hours and frozen at -80 until analyzed per manufacturer's guidelines.

Mice

All animals used in this study, either as a source of primary cells or for *in vivo* analysis, have been previously described. These include animals lacking cGAS*(36)*, STING*(37)*, IFNAR1 (provided by M.K. Kaja and described in *(38)*), TNF (Jax stock #003008), TNFR1 (Jax stock #002818), TNFR2 (Jax stock #002620), DAI(generated in *(39)* and kindly provided by Dr. William Kaiser), AIM2 (Jax stock #013144), Caspase1 and 11 (Generated in *(40)* and kindly provided by Dr. Michael Gale), RIPK3*(41)*, MLKL*(42)*, MAVS (kindly provided by Drs. M. Loo and M. Gale, U. of Washington), TLR3*(43)*, STAT1 (kindly provided by Dr. Herbert W. Virgin) and STAT2 (Jax stock #023309). All animals used were backcrossed to the C57BL/6J background, with the exception of RIPK3 knockouts as described.

DMXAA injection

Animals use for *in vivo* analysis, including wild-type controls, were bred and housed in the UW animal facility. Animals were injected intraperitoneally with 40mg/kg DMXAA (MedKoo) resuspended in 84% PBS, 10% $NaHCO_3$ (5% in H_2O), and 6% DMSO. Weights, surface temperatures, and clinical signs were monitored at 0, 4, 6, 8, 12, and 24 hours post injection. Clinical score was determined by appearance and clinical signs; scores were additive for each sign and animals with cumulative scores of nine or above were euthanized. Sign (score): Lack of grooming (1), ocular discharge (2), hunched (2), duck walk (4), weight loss between 15-20% of starting body weight (4), lethargic (6), weakly responsive or surface temperature less than 28.5° C (8), weight loss greater than 20% of starting body weight, moribund, or surface temperature less than 27.5° C (9). To determine serum cytokines, blood was collected 6 hours post injection by terminal heart puncture. Mice that were used for collection of serum were not included in survival analysis. TNF and IL-6 in serum was measured using ELISA (eBioscience).

Western blots

Macrophages were lysed in buffer containing 20mM Tris HCl (pH 7.5), 135mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 1% Triton X-100, 10% glycerol, and 2X Pierce protease/phosphatase inhibitor mini tablets (Fisher). Thirty micrograms of protein was run on 4%-12% Novex Tris-Glycine gel (Fisher) in running buffer (24mM Tris, 32mM glycine, 3.5mM SDS) and transferred to a PVDF membrane (Thermo) in transfer buffer (6mM Tris, 8mM glycine, 20% methanol). Membranes were blocked overnight in 5%

bovine serum albumin (BSA) in TBS plus 1% Tween20 (TBST). Membranes were incubated overnight with primary antibody in 5% BSA in TBST. Membranes were incubated with HRP-conjugated secondary antibodies for 1 hour and visualized with ECL Western Blotting substrate (Pierce) or (for phospho blots) Femto ECL Western blotting substrate (Pierce). Primary antibodies used: phospho-RIPK3 antibody (Genentech), phospho-MLKL antibody (Abcam, ab196436), total RIPK3 (Imgenex, IMG-5523-2), total MLKL (Clone 3H1), total RIPK1 (BD, 610458), total caspase-8 (Enzo, 1G12), actin (Millipore, MAB1501). Secondary antibodies used: anti-rabbit (Jackson ImmunoResearch), anti-mouse (Santa Cruz), anti-rat (Santa Cruz), and anti-goat (Santa Cruz).

Quantitative RT-PCR

Cells were lysed in RNA Stat 60 (Amsbio). cDNA was synthesized using oligo(dT) and SuperScript III Reverse Transcriptase (Life Tech). Quantitative PCR was performed with SYBR Green (Invitrogen) and a ViiA 7 Real-Time PCR System (Applied Biosystems) using the primers listed below. CT values for each measured gene were normalized to CT values of the housekeeping gene *Gapdh*, and then normalized to a wildtype untreated control sample to obtain ΔΔCT values. Fold change was calculated as 2^(-ΔΔCT) for each sample.

Primers used: *IP-10* forward 5′-AAGTGCTGCCGTCATTTTCTGCCTC-3′, *IP-10* reverse, 5′-CTTGATGGTCTTAGATTCCGGATTC-3′; *Isg15* forward, 5′-GGTGTCCGTGACTAACTCCAT-3′, *Isg15* reverse, 5′-TGGAAAGGGTAAGACCGTCCT-3′; *Ifit1* forward, 5′-GCCATTCAACTGTCTCCTG-3′, *Ifit1* reverse, 5′-

GCTCTGTCTGTGTCATATACC-3′; *Mx1* forward, 5′-GACCATAGGGGTCTTGACCAA-3′, *Mx1* reverse, 5′-AGACTTGCTCTTTCTGAAAAGCC-3′; *Gapdh* forward 5′-GGCAAATTCAACGGCACAGT-3′, *Gapdh* reverse 5′-AGATGGTGATGGGCTTCCC-3′.

Supplementary Materials (4 supplementary figures)

Figure S1: Involvement of STAT1 and STAT2 in necroptosis

Figure S2: Roles of TNFR1 and TNFR2 in necroptosis

Figure S3: Pathways stimulated by 5’ triphosphate RNA (RIG-I ligand) or Poly(I:C)

Figure S4: The effects of STING agonist DMXAA *in vitro* and *in vivo*

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Figure 1

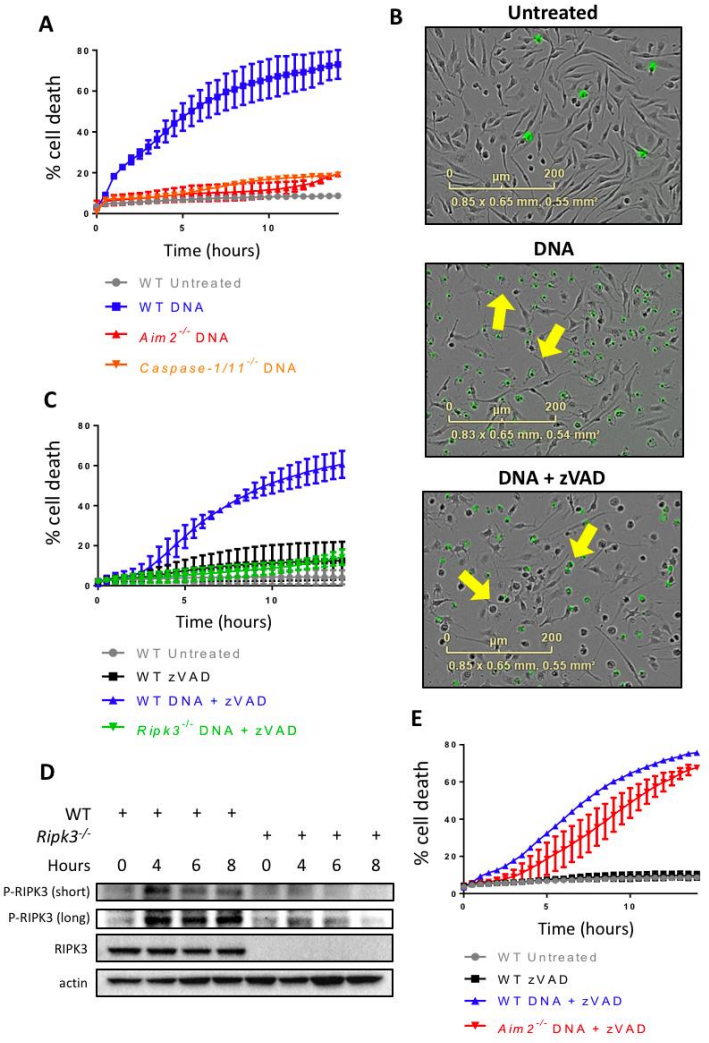

Figure 1: Introduction of DNA into the cytosol can trigger necroptotic cell death. (A) Kinetic cell death of WT, *Aim2*$^{-/-}$, or *Caspase-1/11*$^{-/-}$ primary bone marrow-derived macrophages (BMDM) after transfection with 2 μg/ml DNA. Death measured by uptake of cell impermeable Sytox green dye, and normalized to starting number of cells stained with cell permeable Syto green dye to calculate percent cell death. Error bars represent SD from three independent experiments. (B) IncuCyte images of WT BMDM treated with 2 μg/ml DNA, or 2 μg/ml DNA + 50 μM zVAD. Sytox Green staining is shown in green. (C) Kinetic cell death of WT or *Ripk3*$^{-/-}$ BMDM after treatment with 2 μg/ml cytosolic DNA and 50uM pan-caspase inhibitor zVAD. (D) Western blot analysis of phosphorylated RIPK3 at short and long exposure following treatment of 5 μg/ml cytosolic DNA and 50 μM pan-caspase inhibitor zVAD in WT or *Ripk3*$^{-/-}$ BMDM. (E) Kinetic cell death of WT or *Aim2*$^{-/-}$ BMDM after treatment with 2 μg/ml cytosolic DNA and 50 μM pan-caspase inhibitor zVAD.

Figure 2

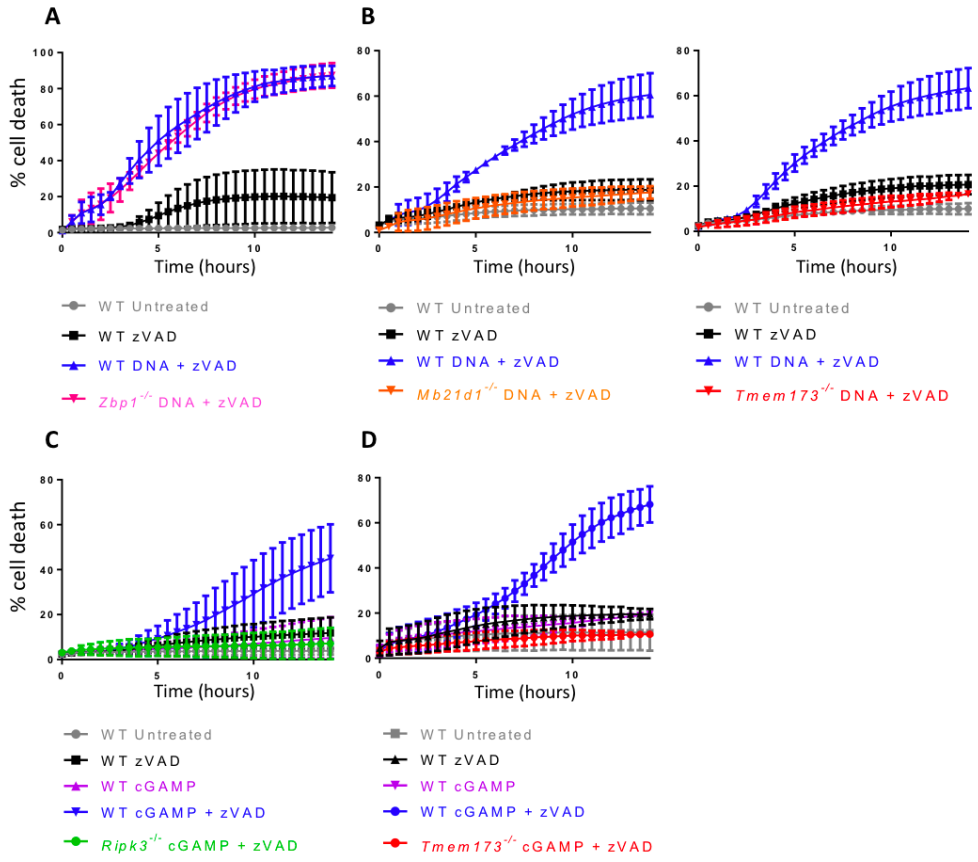

Figure 2: Cytosolic DNA triggers necroptosis via the cGAS-STING pathway. (A) Kinetic cell death of WT or *Zbp1*$^{-/-}$ BMDM after treatment with 2 μg/ml cytosolic DNA and 50 μM pan-caspase inhibitor zVAD. (B) Kinetic cell death of WT, *Mb21d1*$^{-/-}$, *Tmem173*$^{-/-}$ BMDM after treatment with 2 μg/ml cytosolic DNA and 50 μM pan-caspase inhibitor zVAD. (C) Kinetic cell death of WT or *Ripk3*$^{-/-}$ BMDM after treatment with 2 μg/ml cGAMP and 25 μM pan-caspase inhibitor zVAD. (D) Kinetic cell death of WT or *Tmem173*$^{-/-}$ BMDM after treatment with 2 μg/ml cGAMP and 25 μM pan-caspase inhibitor zVAD.

Figure 3

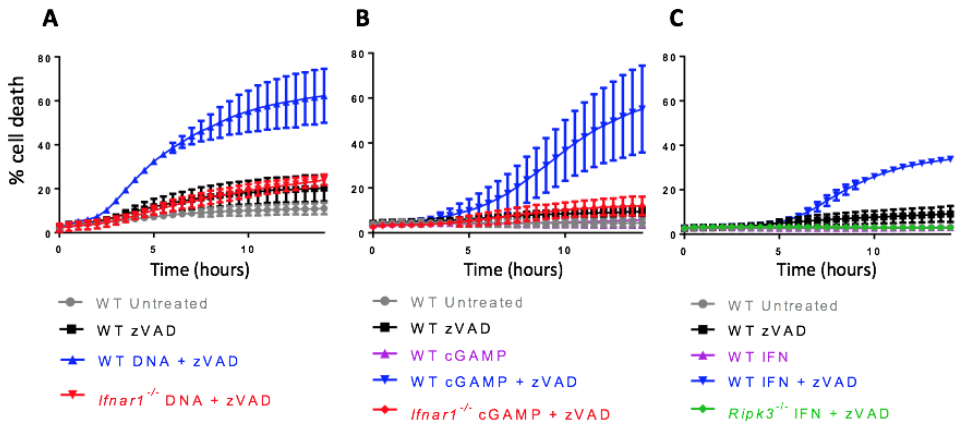

Figure 3: STING-dependent IFN production is necessary but not sufficient to induce necroptosis. (A) Kinetic cell death of WT or *Ifnar1*$^{-/-}$ BMDM after treatment with 2 μg/ml cytosolic DNA and 50 μM pan-caspase inhibitor zVAD. (B) Kinetic cell death of WT or *Ifnar1*$^{-/-}$ BMDM after treatment with 2 μg/ml cGAMP and 25 μM pan-caspase inhibitor zVAD. (C) Kinetic cell death of WT or *Ripk3*$^{-/-}$ BMDM after treatment with 100 units/ml recombinant IFN and 25 μM pan-caspase inhibitor zVAD.

Figure 4

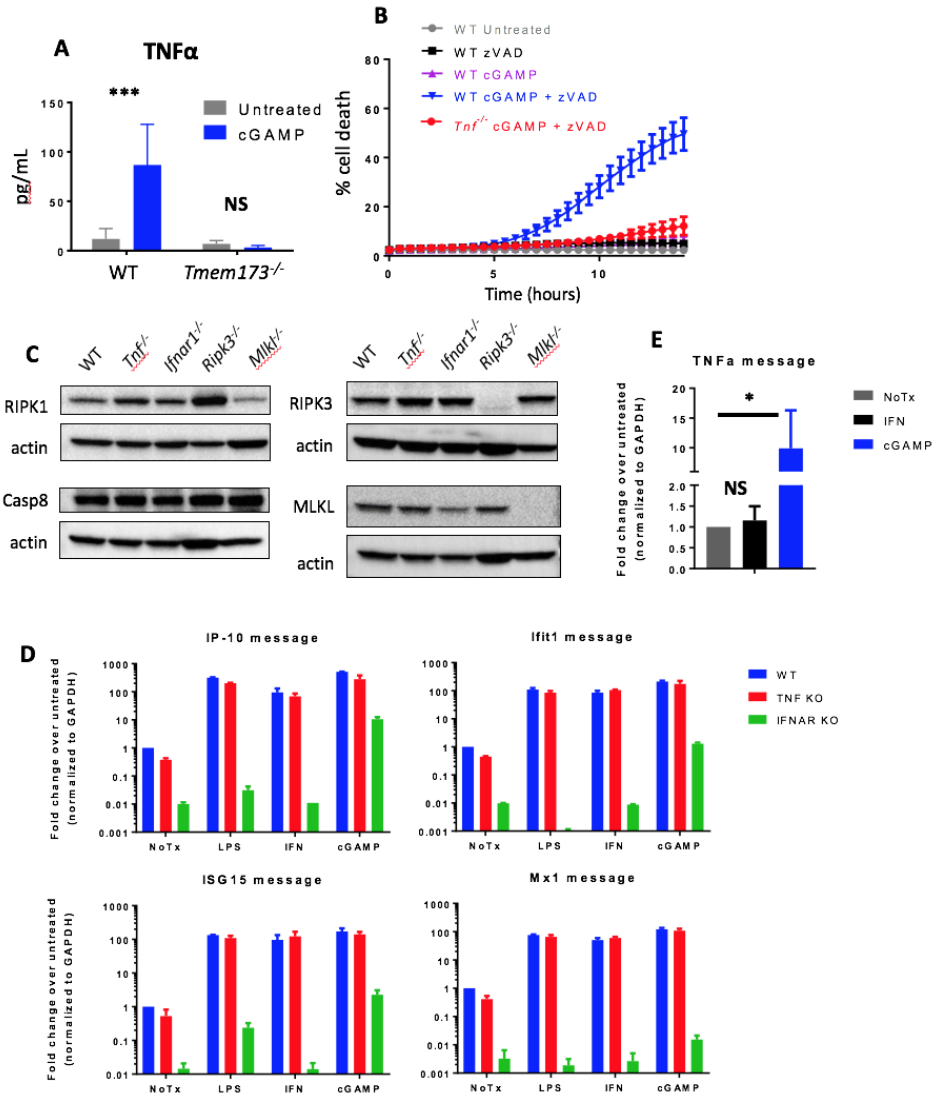

Figure 4: STING-dependent TNF production is required for the induction of necroptosis Luminex analysis of TNFα protein levels in supernatants from WT or *Tmem173*$^{-/-}$ BMDM following treatment with 5 μg/ml cGAMP. Error bars represent SD for three independent experiments. (B) Kinetic cell death of WT or *Tnf*$^{-/-}$ BMDM after treatment with 2 μg/ml cGAMP and 25 μM pan-caspase inhibitor zVAD. (C) Western blot analysis of RIPK1, Caspase-8, RIPK3, and MLKL protein levels at steady state in WT, *Tnf*$^{-/-}$, *Ifnar1*$^{-/-}$, *Ripk3*$^{-/-}$, or *Mlkl*$^{-/-}$ BMDM. (D) Quantitative PCR analysis of interferon stimulated genes (IP-10, Ifit1, ISG15, Mx1) in WT, *Tnf*$^{-/-}$, or *Ifnar1*$^{-/-}$ BMDM following treatment with 200 ng/ml LPS, 100 units/ml recombinant IFN, or 5 μg/ml cGAMP. Error bars represent SD for two independent experiments with three technical replicates. Raw values were normalized to housekeeping gene *Gapdh* and then to the WT untreated sample. (E) Quantitative PCR analysis of *Tnf* in WT BMDM following treatment with 100 units/ml recombinant IFN or 5 μg/ml cGAMP. *p < 0.05, **p < 0.01, ***p < 0.001

Figure 5

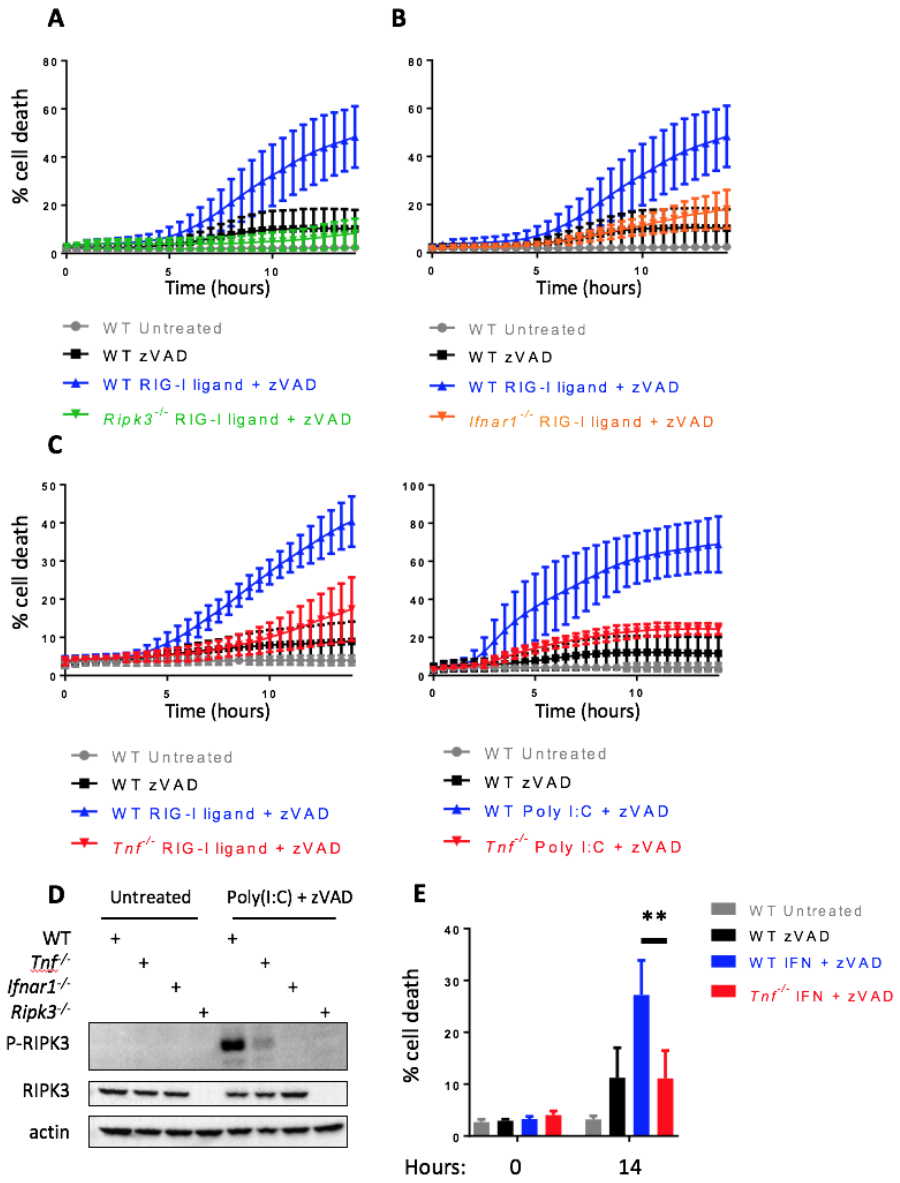

Figure 5: Synergistic TNF and IFN singaling is required for necroptosis downstream of TLR3 and RIG-I. (A) Kinetic cell death of WT or *Ripk3*$^{-/-}$ BMDM after treatment with 1 μg/ml tri-phosphate RNA (RIG-I ligand) and 25 μM pan-caspase inhibitor zVAD. (B) Kinetic cell death of WT or *Ifnar1*$^{-/-}$ BMDM after treatment with 1 μg/ml tri-phosphate RNA (RIG-I ligand) and 25 μM pan-caspase inhibitor zVAD. (C) Kinetic cell death of WT or *Tnf*$^{-/-}$ BMDM after treatment with 1 μg/ml tri-phosphate RNA (RIG-I ligand) and 25 μM pan-caspase inhibitor zVAD or 1 μg/ml poly(I:C) and 25 μM pan-caspase inhibitor zVAD. (D) Western blot analysis of phosphorylated RIPK3 in WT, *Tnf*$^{-/-}$, *Ifnar1*$^{-/-}$, or *Ripk3*$^{-/-}$BMDM following treatment with 2 μg/ml poly(I:C). Cells were harvested 8 hours post treatment. (E) Cell death analysis of WT or *Tnf*$^{-/-}$ BMDM after treatment with 100 units/ml recombinant IFN and 25 μM pan-caspase inhibitor zVAD.

Figure 6

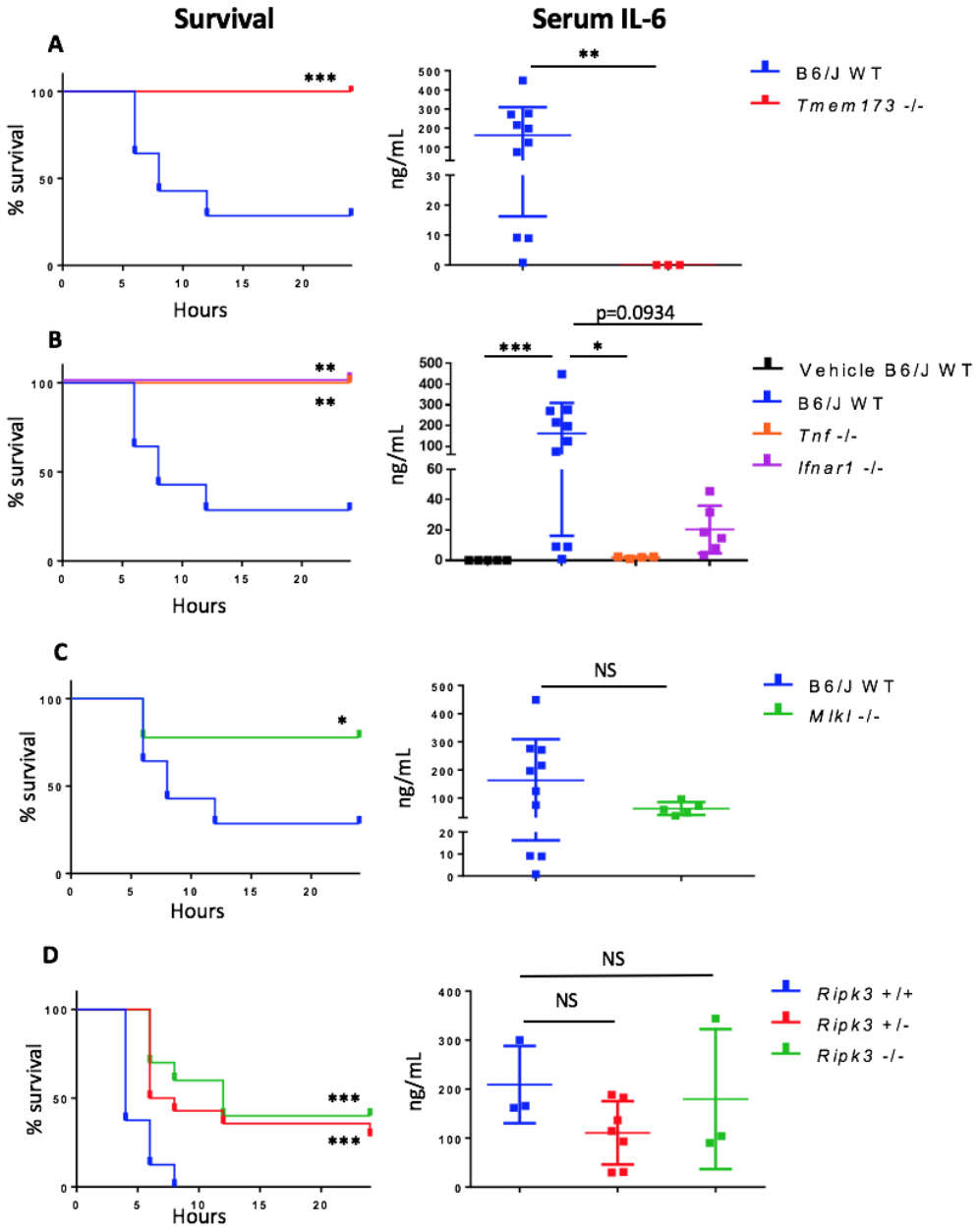

Figure 6: STING agonists induce sterile shock that engages TNF, IFN, and necroptosis *in vivo.* (A) Survival analysis and serum IL-6 of age/sex matched WT C57BL/6J and *Tmem173*$^{-/-}$ mice after intraperitoneal injection of 40 mg/kg DMXAA. Serum was collected 6 hours post treatment. (B) Survival analysis and serum IL-6 of age/sex matched WT C57BL/6J, *Tnf*$^{-/-}$, and *Ifnar1*$^{-/-}$ mice after intraperitoneal injection of 40 mg/kg DMXAA. (C) Survival analysis and serum IL-6 of age/sex matched WT C57BL/6J and *Mlkl*$^{-/-}$ mice after intraperitoneal injection of 40 mg/kg DMXAA. (D) Survival analysis and serum IL-6 of littermate C57BL/6N *Ripk3*$^{+/+}$, *Ripk3*$^{+/-}$, *Ripk3*$^{-/-}$ mice after intraperitoneal injection of 40 mg/kg DMXAA. *p < 0.05, **p < 0.01, ***p < 0.001. All data is pooled from three or more independent experiments.

Supplemental Figure 1

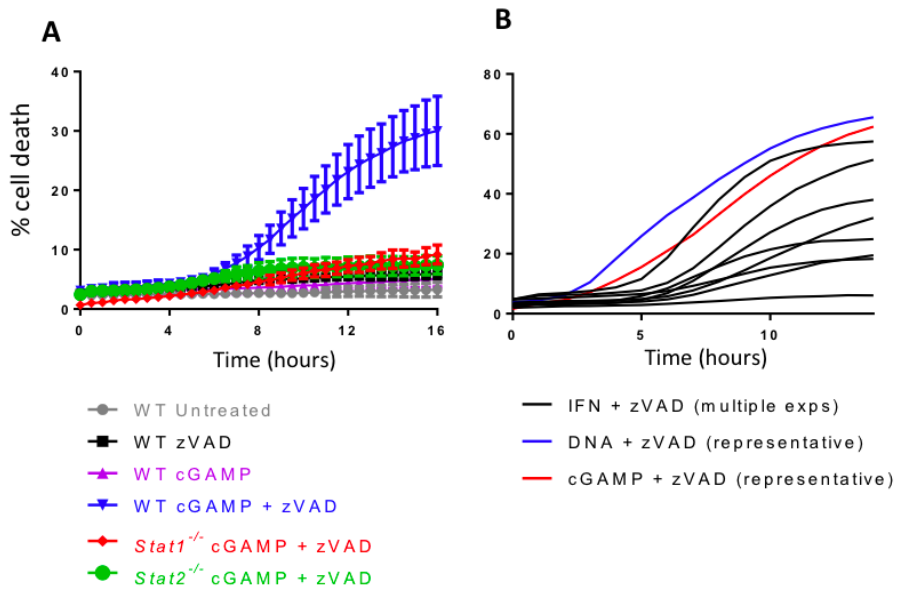

Figure S1: Involvement of STAT1 and STAT2 in necroptosis (A) Kinetic cell death of WT, *Stat1*$^{-/-}$, or *Stat2*$^{-/-}$ BMDM after treatment with 2 μg/ml cGAMP and 25 μM pan-caspase inhibitor zVAD. (B) Kinetic cell death of WT BMDM following treatment with 100 units IFN and 25 μM zVAD, 2 μg/ml cytosolic DNA and 50 μM zVAD, or 2 μg/ml cGAMP and 25 μM zVAD. Black lines are individual experiments, representative of the variability observed upon stimulus with recombinant IFN. Blue line and red line are one experiment of the representative average of response.

Supplemental Figure 2

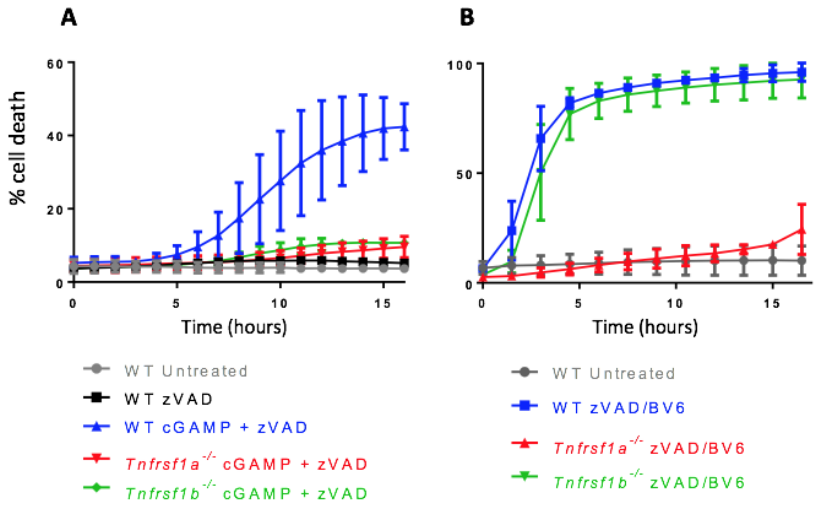

Figure S2: Roles of TNFR1 and TNFR2 in necroptosis (A) Kinetic cell death of WT, *Tnfrsf1a*$^{-/-}$, or *Tnfrsf1b*$^{-/-}$ BMDM after treatment with 2 μg/ml cGAMP and 25 μM pan-caspase inhibitor zVAD. (B) Kinetic cell death of WT, *Tnfrsf1a*$^{-/-}$, or *Tnfrsf1b*$^{-/-}$ BMDM after treatment with 1 μM cIAP antagonist BV6 and 25 μM pan-caspase inhibitor zVAD.

Supplemental Figure 3

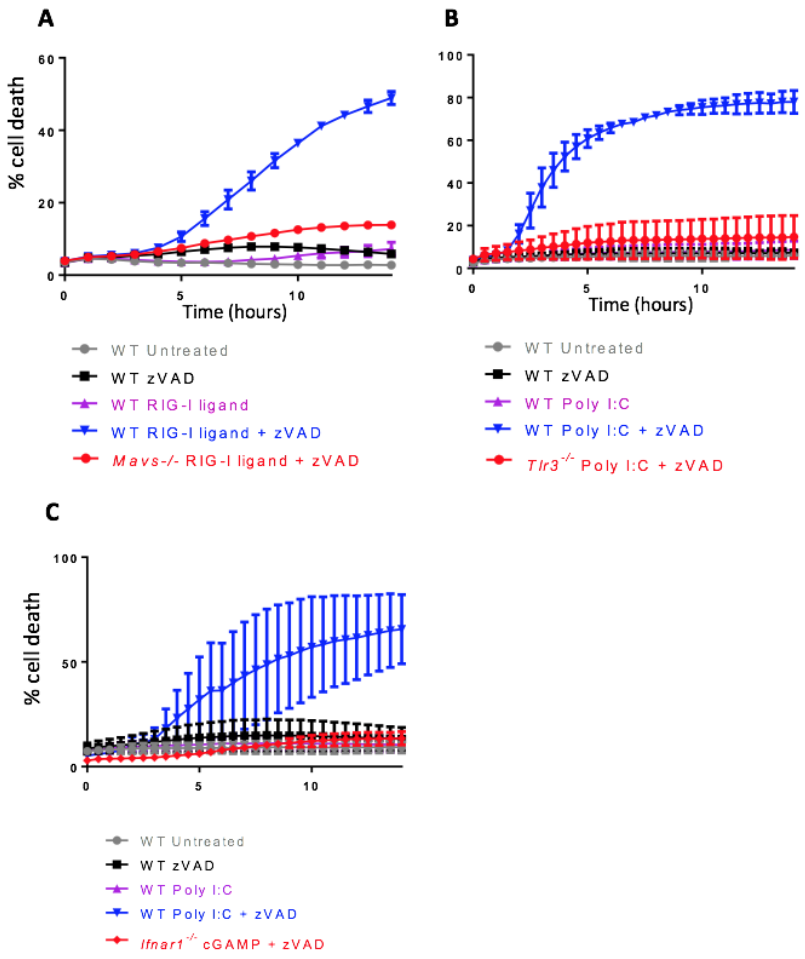

Figure S3: Pathways stimulated by 5' triphosphate RNA (RIG-I ligand) or Poly(I:C) (A) Kinetic cell death of WT or *Mavs*$^{-/-}$ BMDM after treatment with 1 μg/ml tri-phosphate RNA (RIG-I ligand) and 25 μM pan-caspase inhibitor zVAD. (B) Kinetic cell death of WT or *Tlr3*$^{-/-}$ BMDM after treatment with 1 μg/ml poly(I:C) and 25 μM zVAD. (C) Kinetic cell death of WT or *Ifnar1*$^{-/-}$ BMDM after treatment with 1 μg/ml poly(I:C) and 25 μM zVAD.

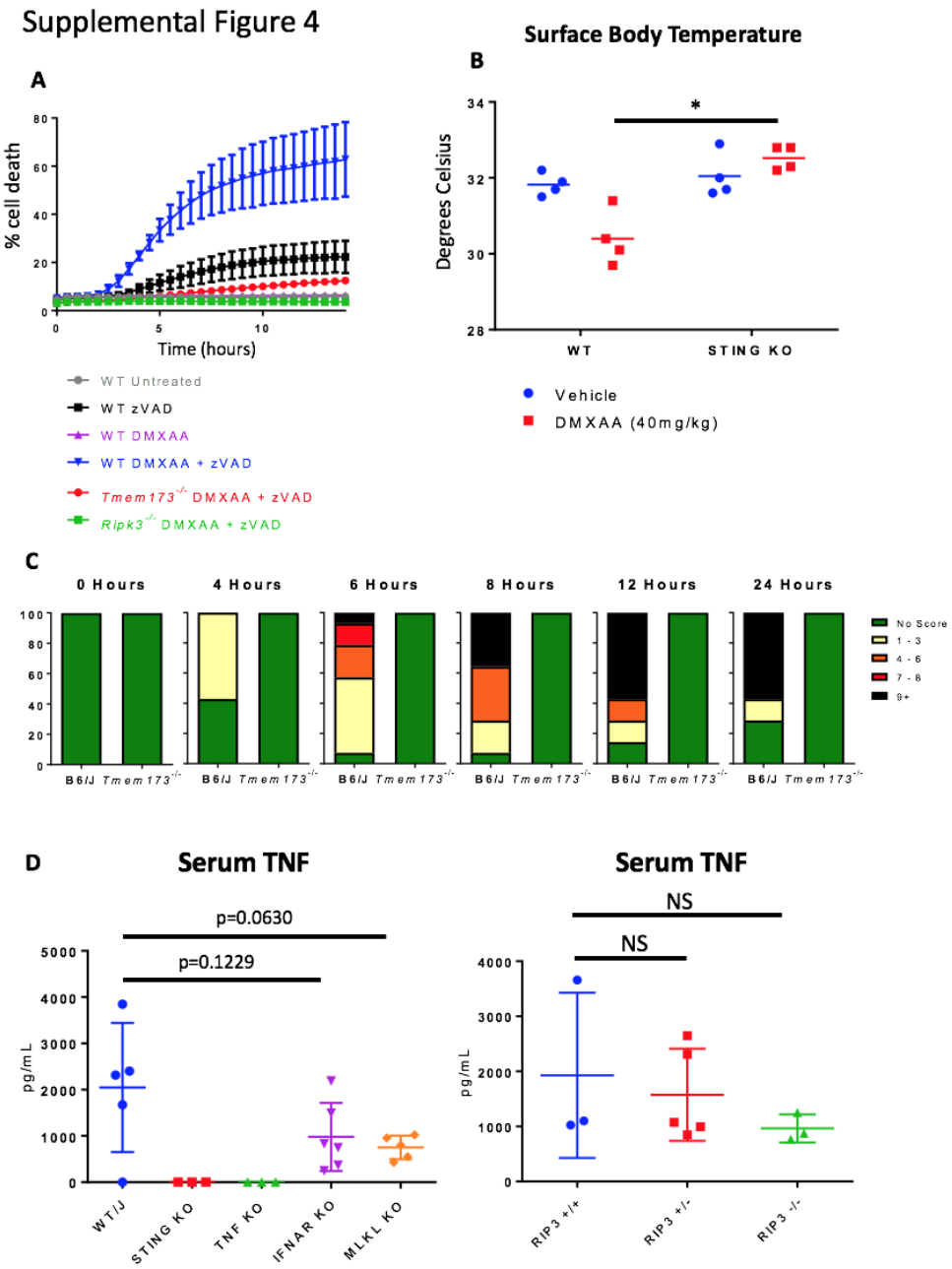

Figure S4: The effects of STING agonist DMXAA *in vitro* and *in vivo* (A) Kinetic cell death of WT, *Tmem173*$^{-/-}$, or *Ripk3*$^{-/-}$ BMDM after treatment with 30 μg/ml DMXAA and 25 μM pan-caspase inhibitor zVAD. (B) Surface body temperature of WT or *Tmem173*$^{-/-}$ mice following IP injection of 40 mg/kg DMXAA. Temperatures are an average of three readings taken 8 hours post treatment. (C) Clinical scores of WT or *Tmem173*$^{-/-}$ mice following IP injection of 40 mg/kg DMXAA. Scores were cumulative based on signs as listed in Methods. (D) Serum TNF measured by ELISA of WT, *Tmem173*$^{-/-}$, *Tnf*$^{-/-}$, *Ifnar1*$^{-/-}$, *Mlkl*$^{-/-}$, or *Ripk3* littermates following IP injection of 40 mg/kg DMXAA. Serum was collected 6 hours post injection.

Chapter 4: Conclusions and Future Directions

The data included within my manuscript clarify many gaps in our understandings of links between nucleic acid sensing and necroptosis. First, it shows that DNA itself is capable of triggering necroptosis. This becomes important in cell types where pyroptosis, another lytic form of cell death, is not active or when caspases are suppressed. Activation of RIPK3 by DNA requires the cGAS-STING pathway, not inflammasome or DAI signaling. Upon stimulation of the cGAS-STING pathway, both TNF and IFNAR are produced, and while the production of these cytokines has been noted before, their ability to induce necroptosis after STING activation has now been empirically demonstrated. As STING-deficient mice are susceptible to numerous DNA viruses, and RIPK3 activation would likely be defective in these animals, perhaps part of the reason for the susceptibility to these viruses is due to the inability to remove infected cells by necroptosis. New tools have recently become available that may allow for determination of activation status within infected tissue in murine models, namely a reliable phospho-RIPK3 antibody, which would allow for greater interrogation of the role of necroptosis in DNA infection *in vivo*.

Clearly DNA-induced necroptosis does play an important role *in vivo* as our data show over-activation of STING has detrimental effects in mice. Presumably over-activation of STING in humans who are currently in immunotherapy clinical trials with STING agonists might also result in toxicity. An important next step will be determining whether the efficacy of these agonists requires RIPK3 activation. As necroptotic cells have been shown to more efficiently cross present antigens[15], RIPK3 could play an important role in clearing tumors following STING activation. However, if RIPK3 were

not an important player in the efficacy of STING agonists to promote tumor clearance, the addition of a RIPK3-blocking drug could potentially reduce off-target toxicity and allow for increasing doses of agonist. As there is already a RIPK1/RIPK3-blocking, clinically approved drug available[47], modulating necroptosis could be an easy and effective way to maximize the clinical benefit of STING agonists. However, the role that necroptosis plays in tumor clearance must be more robustly determined.

While DMXAA treatment *in vitro* requires caspase inhibition to see appreciable necroptosis, treatment *in vivo* does not require a caspase inhibitor. This has been noted with TNF treatment[48], and viral infections in which the virus does not encode a caspase inhibitor, such as influenza. Intriguingly, influenza infection *in vitro* also does not require a caspase inhibitor to see RIPK3-dependent death, making it one of the only *in vitro* treatments that does not require caspase inhibition. As discussed in the review in Chapter 2, perhaps there are additional "trapdoors" that treatments or infections can trigger in a whole organism in addition to caspase inhibition that sensitize cells to necroptosis. As influenza can trigger this death *in vitro* as well, it could be a helpful tool to identify additional ways that pathogens can sensitize cells to necroptosis. Investigating changes in transcription, translation, or perhaps even metabolism of cells infected with influenza *in vitro* could potentially help identify other "trapdoors" that might help trigger necroptosis in infections within a whole organism. Understanding when and how necroptosis occurs in infection could ultimately lead to better viral control, whether it be by designing more effective vaccinations or treatments that harness the inherently anti-viral nature of necroptosis.

In addition to STING agonists, RIG-I and TLR agonists are being investigated as potential broad spectrum anti-virals or vaccine adjuvants[49], since they induce high levels of IFN which can protect against numerous viral infections. As we have seen that RIG-I and TLR3 activation can activate RIPK3 in a manner similar to STING activation, requiring IFN and TNF, presumably agonists of these pathways might have similar risks of toxicity at high doses. Understanding these risks and potentially modulating the necroptotic pathway to minimize off target effects will be important moving forward.

Finally, while we determined that both IFN and TNF are required for the induction of necroptosis, it is still unclear how these signals interact. Intriguingly, TNF is required for IFN-induced necroptosis, however TNF is not upregulated upon IFN treatment. This eliminates the simplest answer that TNF upregulation itself is responsible for activation of RIPK3 following IFN treatment. There are many layers of regulation of TNF signaling, such as the availability of TNF receptors on the cell surface, and the activity of the TNF converting enzyme (TACE) on the cell surface to allow for cleavage of TNF into its soluble form. Even further, it is unclear whether the TNF signal required to activate RIPK3 following nucleic acid sensing or IFN signaling is from soluble TNF or membrane bound TNF. During the course of these studies, many of these ideas were tested – including availability of TNF receptors on the cell surface in WT and IFNAR-deficient cells and upregulation of TACE by IFN signaling. In my hands, nothing definitive was discovered that would explain how IFN might regulate these processes, and therefore how IFN and TNF were synergizing to induce necroptosis. However, these processes and other key regulator processes of TNF signaling could be interrogated more thoroughly to determine the exact mechanism of synergy between TNF and type I IFN

to induce RIPK3-dependent cell death. Overall, this research further cements necroptosis as one important outcome of signaling following nucleic acid signaling, which has implications for the future of antiviral therapies, as well as tumor immunotherapy.

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(Note: References below are for Chapters 1 and 4 only. Chapters 2 and 3 are publications with their own internal references)

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